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Application Note

Superior lot-to-lot consistency of cytokine measurement in human serum and plasma with a MILLIPLEX® multiplex immunoassay

Introduction

Multiplex immunoassays are valuable research tools for studying circulating cytokines, chemokines, and growth factors. Lot-to-lot consistency is an important requirement when analyzing biomarkers, with longitudinal studies of immune factors providing insight into inflammation and disease. When lot-to-lot performance is not consistent in immunoassays, longitudinal studies are not possible as changes in assay performance such as sample drift can produce confounding or misleading results leading to incorrect conclusions. In this study, we focused on two immunoassay performance indicators related to consistency: standard curve reproducibility and sample measurement.

The lot-to-lot performance of the MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A (Cat. No. HCYTA-60K) 48-plex immunoassay was evaluated and compared to similar assays from leading competitors, referred to here as Brand X, Brand Y, and Brand Z, using matched human serum and plasma from healthy donors. Brand X and Brand Z are 45-plex immunoassays and Brand Y is a 48-plex immunoassay. Each assay uses magnetic microspheres from Luminex® technology to simultaneously measure up to 48 cytokines. Two different lots from each kit were tested with the same matched sample cohort and data were analyzed with Belysa® Immunoassay Curve Fitting Software (Cat. No. 40-122). All four of the kits share an overlapping set of 27 analytes, including: Eotaxin/CCL11, FGF-2, GM-CSF, GROα, IFNα, IFNγ, IL-1α, IL-1β, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-15, IL-17A/CTLA8, IP-10/CXCL10, MCP-1, MIP-1α, MIP-1β, RANTES, TNFα, and VEGF-A.

Methods

Sample Preparation

Seven matched human plasma and serum samples were obtained from healthy donors from a commercial vendor (BioIVT, Westbury, NY). 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 prior to use, to remove particulates. Samples were run neat or diluted, according to the respective kit protocols.

Immunoassays and Data Analysis

Multiplex immunoassays were performed in 96-well plates according to each respective product manual. Two different kit lots were obtained from each vendor over the course of a 2-year period and were each analyzed according to the manufacturers' protocols. All kits were run on the Luminex® 200™ instrument and data was acquired via xPONENT® v. 4.3 software. Standard curve information was obtained from the MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A protocol and the lot-specific Certificates of Analysis for the Brand X, Brand Y, and Brand Z kits. Data analysis was performed for all immunoassays using the Belysa® Curve Fitting Software (Cat. No. 40-122). Figures were prepared in GraphPad Prism and Microsoft Excel.

Results

Standard Curve Reproducibility

Standard curves were generated from each kit lot using averaged mean fluorescence intensity (MFI) from duplicate wells. Standard curve consistency is illustrated by overlaying the curves from both lots. Six representative analytes, shared by all four kits, are displayed in **Figure 1**. Notably, the MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A maintains a consistent standard curve range from one kit lot to another. In comparison, kits from Brand X, Brand Y, and Brand Z have value-assigned standards, such that each kit lot will have a different standard curve range for every analyte. For those reasons, the standard curves from the other three kits appear slightly staggered along the x-axis.

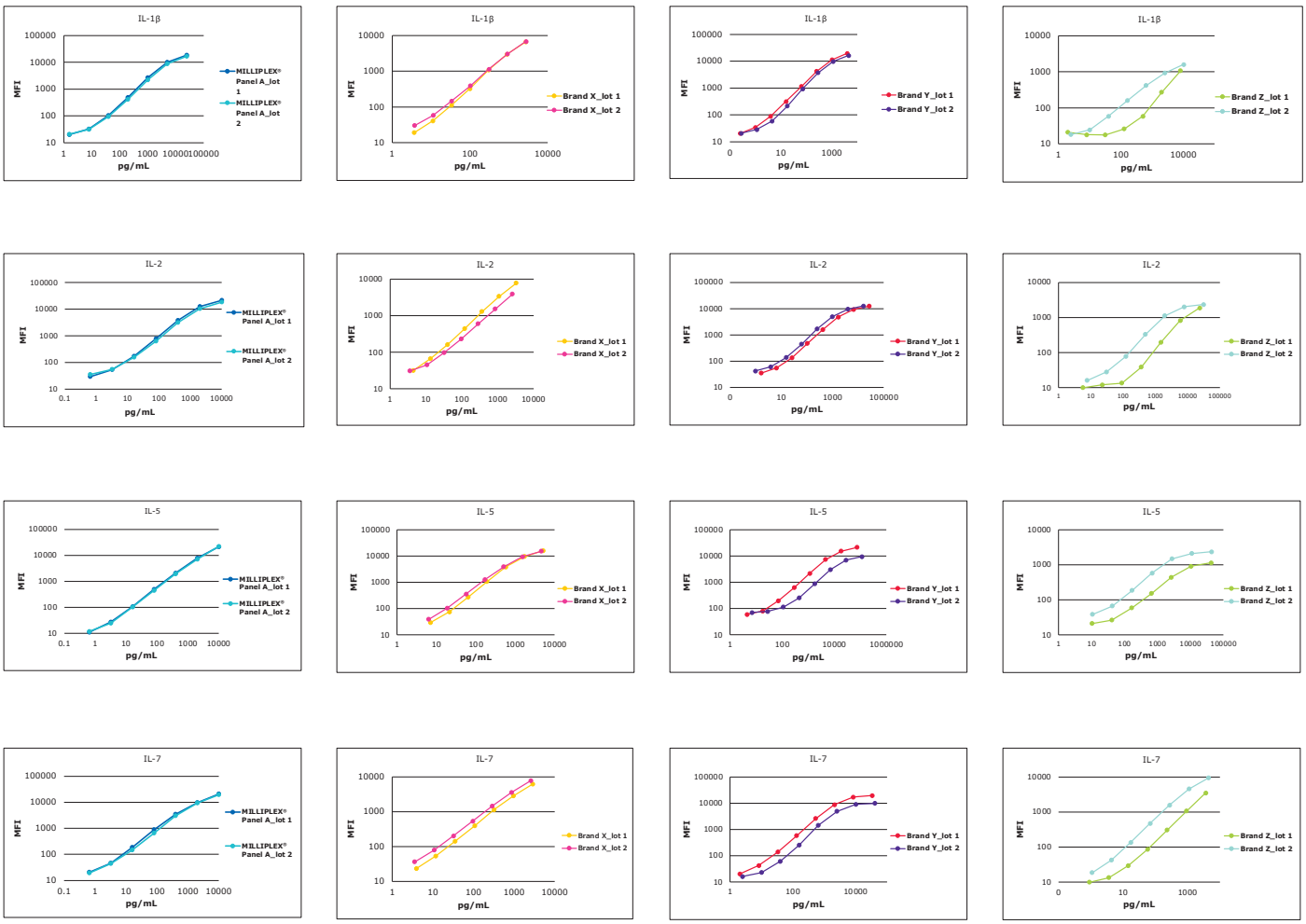

Figure 1. Comparative standard curve performance between two kit lots for MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A and Brand X, Brand Y, and Brand Z.

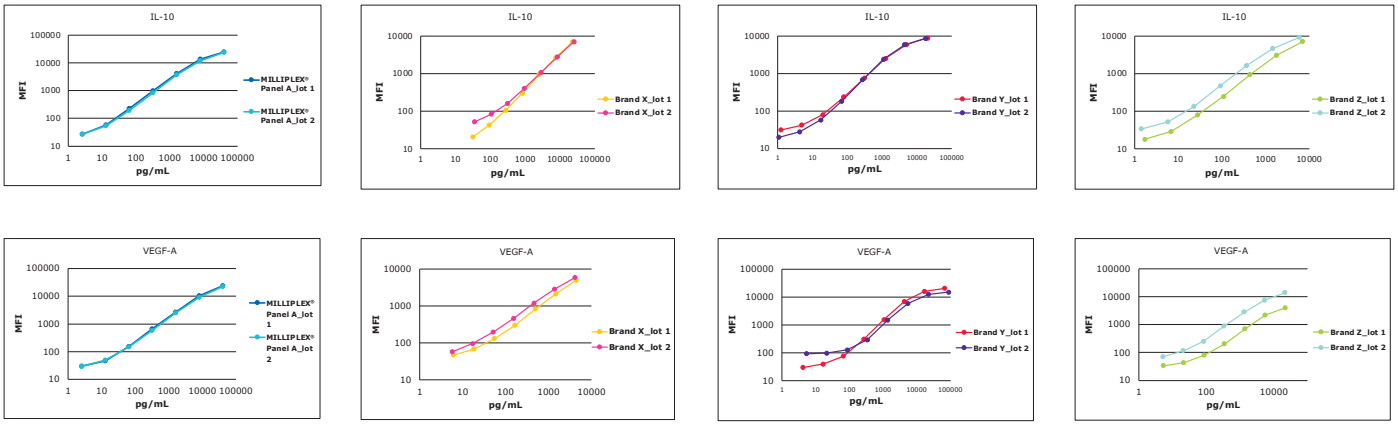

Figure 1 (continued). Comparative standard curve performance between two kit lots for MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A and Brand X, Brand Y, and Brand Z.

Relative potency was then determined using Belysa® Immunoassay Curve Fitting Software. MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A had a median relative potency of 0.80, compared to median relative potencies of 1.90, 0.669, and 3.09 for Brands X, Y, and Z, respectively. A chart of relative potency is shown for analytes common between the four kits in **Figure 2**.

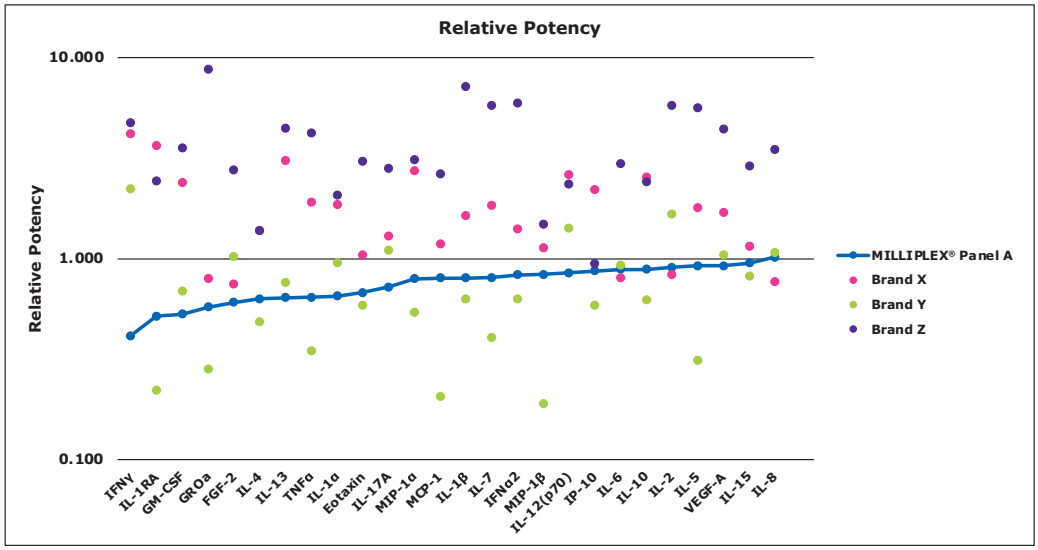

Figure 2. Relative Potency as determined by Belysa® Curve Fitting software for the common analytes in MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A, Brand X, Brand Y, and Brand Z.

Limit of Detection

Limit of Detection (LOD) provides a key indication of assay sensitivity, as the lowest concentration of an analyte that can be reliably detected from background noise. For each analyte the LOD was determined by Belysa® Curve Fitting software using the following equation:

LOD = 3 x σ (blank sample) / $Slope_{II}$, where $Slope_{II}$ is the slope of the least squares regression line that best fits the first three standard groups with increasing concentrations starting from the blanks.

Because this calculation is dependent on the variation of the blank, LOD cannot be calculated when the MFI is identical between duplicate blank samples. Therefore, the LOD is listed as "N/A" for standard curves without variation in their blanks. Users may choose to run standard curves in triplicate to increase the chances of obtaining a tangible LOD value for each analyte.

For each of the four bead-based immunoassays tested, the LOD values for the 27 common analytes were compared between each lot and averaged to

determine a mean LOD value. Data from 26 analytes from MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A, 25 analytes each from Brand X and Brand Z, and 27 analytes from Brand Y were incorporated in the analysis, with analytes only omitted if the MFI in the duplicate background wells was identical in the runs from each lot, thereby producing a LOD of "N/A".

The greatest sensitivity, on average, was observed with MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A, which showed the lowest mean LOD values for the shared analytes; 2.7 pg/mL for the MILLIPLEX® multiplex kit versus 5.6 pg/mL for Brand X, 7.1 pg/mL for Brand Y, and 35.0 pg/mL for Brand Z (**Figure 3A**). Several examples of analyte-specific LOD values are displayed in **Table 1**. The %CV of the LOD (**Figure 3B**) gives an indication of the consistency at the lower end of the curve. As described above, this value could not be obtained for all analytes since runs with identical background MFIs had indeterminate LODs. Thus, only analytes where both runs produced a tangible LOD could be used to calculate the %CV. This included 18 analytes from MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A, 17 analytes from Brand X, 15 analytes from Brand Y, and 13 analytes from Brand Z. The only kit for which the mean %CV value for the LOD for all eligible analytes was less than 50% was MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A.

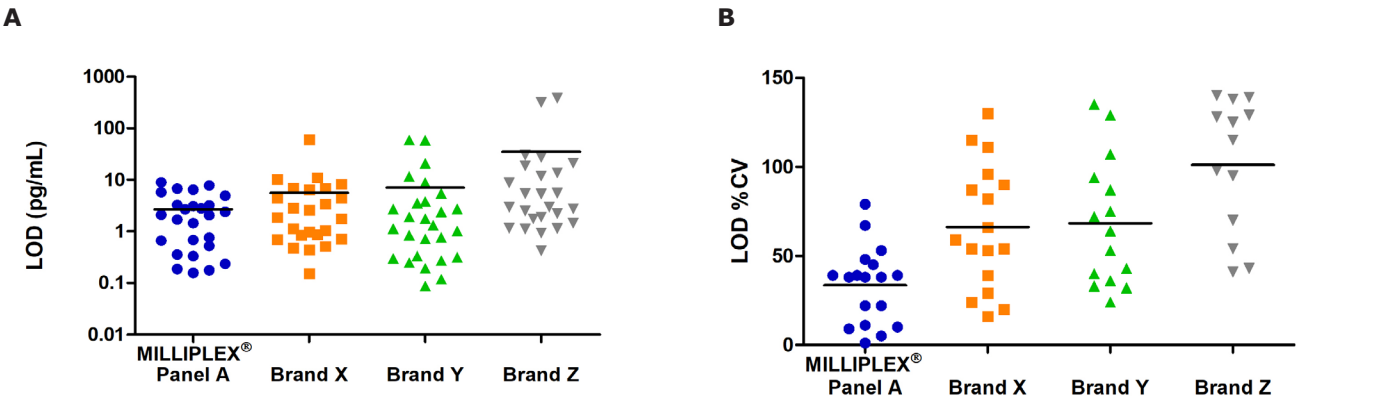

Figure 3. Limit of Detection variation for **(A)** values in pg/mL and **(B)** %CV between kit lots as determined by Belysa® Curve Fitting software for common analytes in MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A, Brand X, Brand Y, and Brand Z.

Limit of Detection (pg/mL)

	MILLIPLEX® Human Panel A (48-plex)	Brand X (45-plex)	Brand Y (48-plex)	Brand Z (45-plex)
GROα	0.24	6.77	58.61	1.92
IL-2	0.19	0.97	0.77	20.98
IL-6	0.68	1.14	0.25	318.09
IL-7	0.16	0.70	2.74	0.93
IL-8	0.36	0.48	0.33	27.17
IL-10	0.76	10.16	1.77	2.53
IL-15	1.44	0.44	20.85	5.54
IL-17A/CTLA8	0.66	0.85	1.32	8.82
MCP-1	2.70	1.04	3.82	5.46
VEGF-A	1.70	4.43	59.04	2.93

Table 1. Limit of Detection (LOD) shown for 10 common analytes in MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A, Brand X, Brand Y, and Brand Z. Green highlighted cells indicate the lowest LOD for each analyte. Yellow highlighted cells indicate LOD values that are more than 10x greater than the lowest LOD.

Sample Detectability

Healthy donor serum and EDTA plasma samples were measured on two occasions with the two lots of each kit. We assessed the ratio of sample values between lots to determine which kit gave the most consistent sample measurement (**Figure 4A**). Additionally, we looked at sample correlation between each lot, with R^2 values generated in Excel (**Figure 4B**). For each metric, the closer the analytes cluster to the value of "1", the better the sample ratio or correlation. Direct comparison of MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A with Brand X (**Figure 4C**), Brand Y (**Figure 4D**), and Brand Z (**Figure 4E**) is also shown with sample ratios plotted on the x-axis and R^2 values on the y-axis. Three examples of sample correlation results between kit lots are shown for IL-13 (**Figure 4F**), MIP-1β (**Figure 4G**), and VEGF-A (**Figure 4H**). We defined minimally satisfactory measurement as concentration value ratios between 0.4 and 2.5. Satisfactory R^2 values for sample correlation between lots were set at or above 0.7. For MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A, all 48 analytes fulfilled at least one of these 2 criteria. The best performing competitor kit only met either the ratio or R^2 criteria for 37 of 45 analytes (**Table 2**). Forty of 48 analytes (83%) in MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A surpassed both benchmarks, with the next best kit achieving both conditions for 29 of 45 analytes (64%).

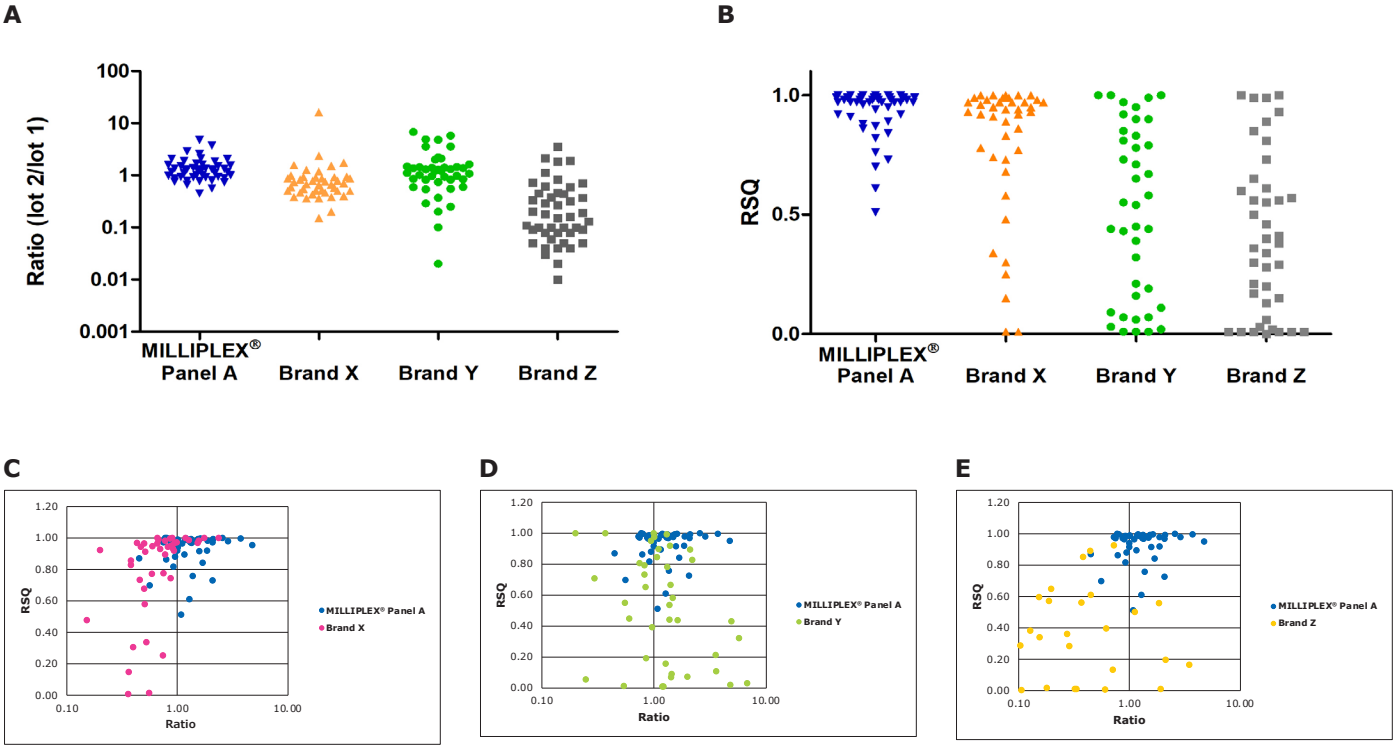

Figure 4. Consistency of sample measurement and correlation between 2 kit lots of MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A compared to Brand X, Y, and Z. **A.** Ratios of sample values between kit lots were used to determine which kit gave the most consistent sample measurement. **B.** R^2 values sample correlation between each kit lot. **C, D, E.** Direct comparison of MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A with **(C)** Brand X, **(D)** Brand Y, **(E)** and Brand Z with sample ratios plotted on the x-axis and R^2 values on the y-axis.

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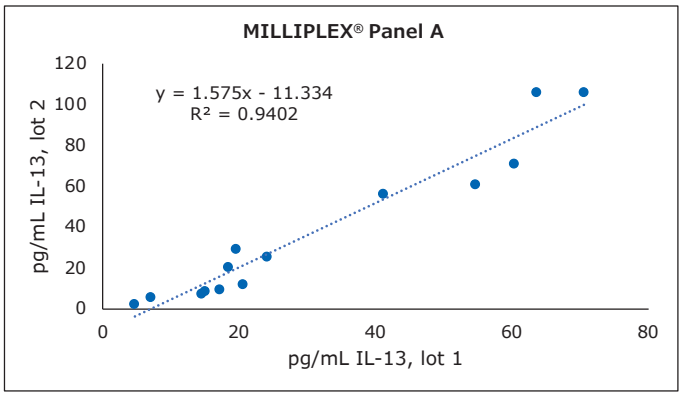

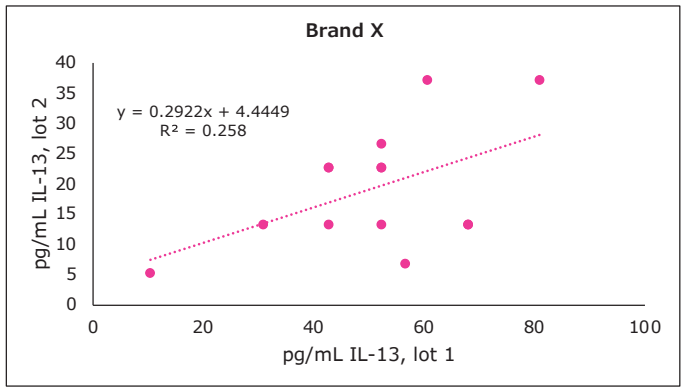

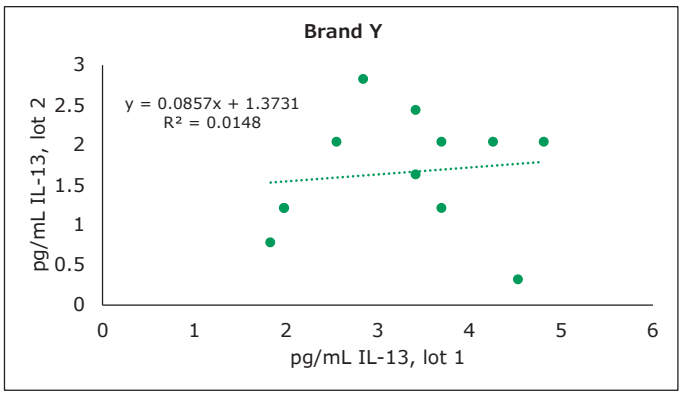

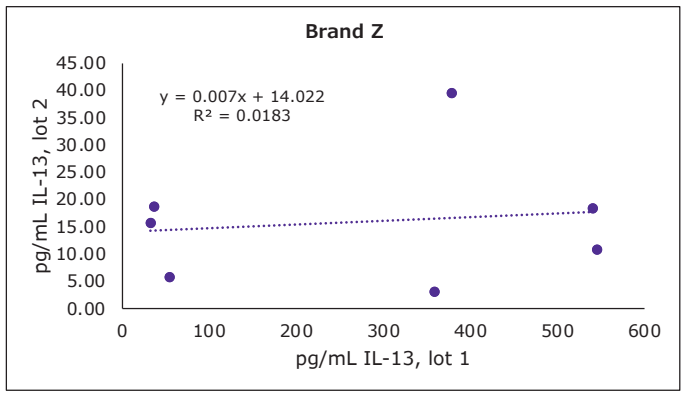

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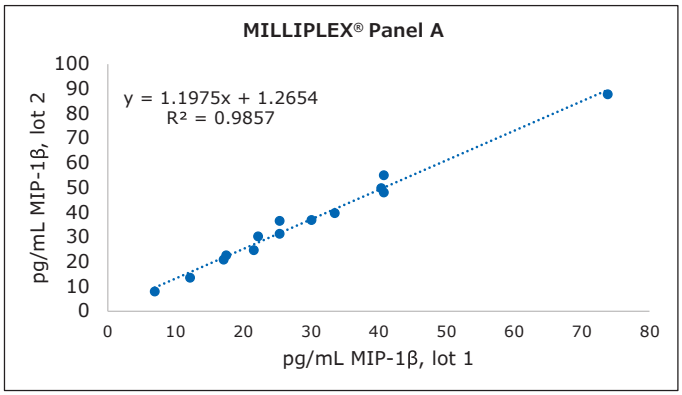

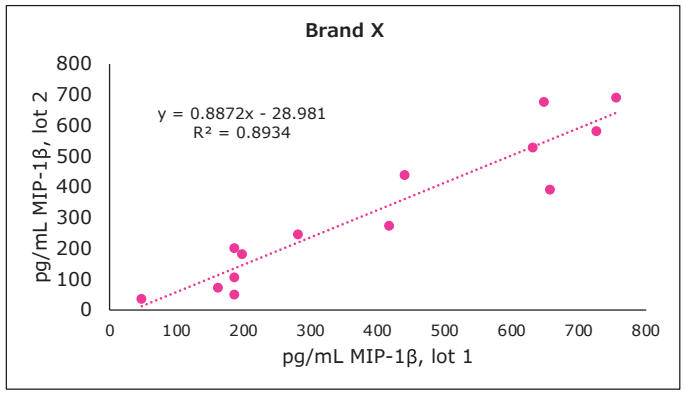

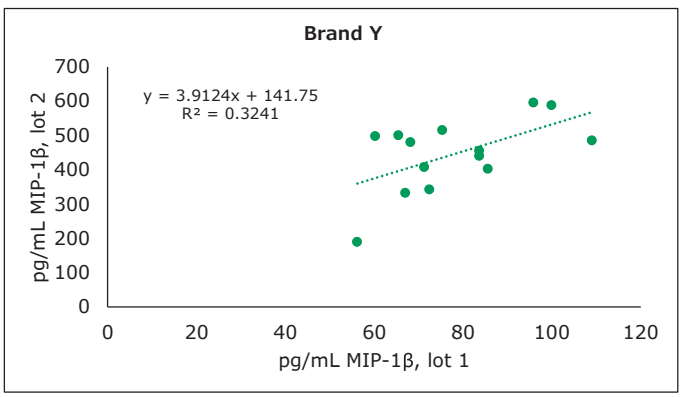

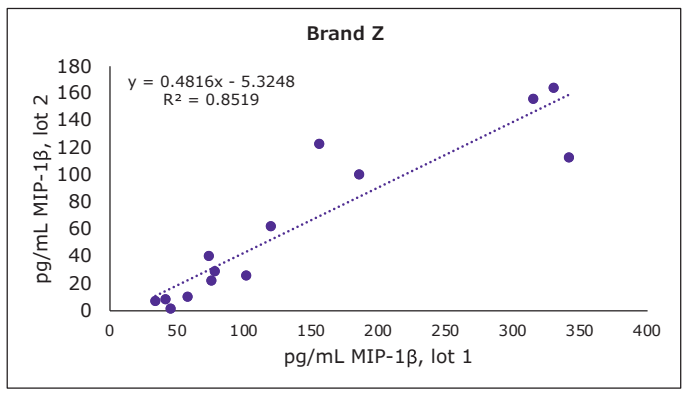

Figure 4. Consistency of sample measurement and correlation between 2 kit lots of MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A compared to Brand X, Y, and Z. **F, G.** Sample correlation results between kit lots for **(F)** IL-13 and **(G)** MIP-1β.

H

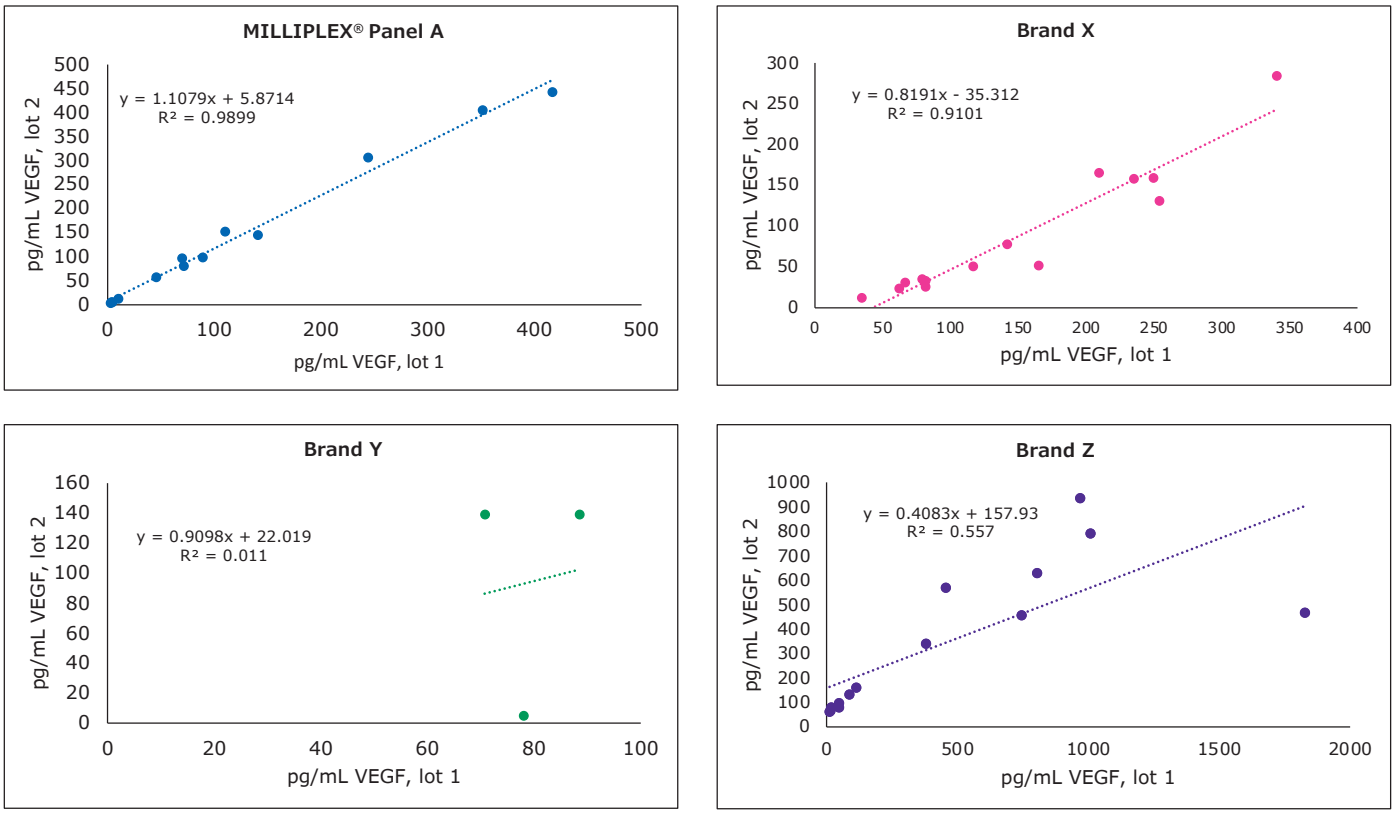

Figure 4. Consistency of sample measurement and correlation between 2 kit lots of MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A compared to Brand X, Y, and Z. **H.** Sample correlation results between kit lots for VEGF-A.

Kit Name	No. of analytes within ratio (0.4-2.5) (%)	No. of analytes with R^2 ≥0.7 (%)	No. of analytes meeting at least 1 criterion (%)	No. of analytes meeting both criteria (%)
MILLIPLEX® Human Panel A (48-plex)	44 (92%)	44 (92%)	48 (100%)	40 (83%)
Brand X (45-plex)	34 (76%)	32 (71%)	37 (82%)	29 (64%)
Brand Y (48-plex)	31 (65%)	16 (33%)	34 (71%)	13 (27%)
Brand Z (45-plex)	12 (27%)	9 (20%)	19 (42%)	2 (4%)

Table 2. Sample performance across two lots of MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A and Brand X, Y, and Z.

Summary

The data presented in this application note demonstrates the reliability of MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A for consistent assay performance, sensitivity, and reproducible measurement of immune factors in serum and plasma between different kit lots. As part of the production and quality testing process, the MILLIPLEX® standards and quality controls are compared to previous lots and a reference lot, our “gold standard,” to ensure lot-to-lot consistency. All data is compiled in trend charts to track lot-to-lot performance. Measuring against a gold standard, in addition to the previous lot standard, is important to control for assay drift that can occur as new lots are produced. Maintaining stringent standard curve potency acceptance criteria is important because it preserves lot-to-lot kit performance enabling reliable assay sensitivity and sample measurement.

Acknowledgments

Thank you to Anthony Saporita[1], PhD and Brooke Gilliam[1] for writing the application note, and to Hong Luo[1], PhD, Harold Steiner[1], Irene Zollars[1], PharmD, Danielle Pepin[1], and Lawrence Rentoul[1] for their thoughtful reviews.

[1]MilliporeSigma, St. Louis, MO USA

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