

Comparison of Multiplex Immunoassay Platforms

Qin Fu,^{1*} Jie Zhu,¹ and Jennifer E. Van Eyk^{1,2,3}

Departments of ¹ Medicine, ² Biomedical Engineering, and ³ Biological Chemistry, Bayview Proteomics Center, Johns Hopkins University, Baltimore, MD; * address correspondence to this author at: Department of Medicine, Division of Cardiology, Bayview Proteomics Center, Johns Hopkins University, 5200 Eastern Ave., Baltimore, MD 21224. E-mail qfu1@jhmi.edu.

BACKGROUND: Candidate biomarkers discovered with high-throughput proteomic techniques (along with many biomarkers reported in the literature) must be rigorously validated. The simultaneous quantitative assessment of multiple potential biomarkers across large cohorts presents a major challenge to the field. Multiplex immunoassays represent a promising solution, with the potential to provide quantitative data via parallel analyses. These assays also require substantially less sample and reagents than the traditional ELISA (which is further limited by its ability to measure only a single antigen). We have measured the reproducibility, reliability, robustness, accuracy, and throughput of commercially available multiplex immunoassays to ascertain their suitability for serum biomarker analysis and validation.

METHODS: Assay platforms MULTI-ARRAY (Meso Scale Discovery), Bio-Plex (Bio-Rad Laboratories), A² (Beckman Coulter), FAST Quant (Whatman Schleicher & Schuell BioScience), and FlowCytomix (Bender MedSystems) were selected as representative examples of technologies currently used for high-throughput immunoanalysis. All assays were performed according to protocols specified by the manufacturers and with the reagents (diluent, calibrators, blocking reagents, and detecting-antibody mixtures) included with their kits.

RESULTS: The quantifiable interval determined for each assay and antigen was based on precision (CV < 25%) and percentage recovery (measured concentration within 20% of the actual concentration). The MULTI-ARRAY and Bio-Plex assays had the best performance with the lowest limits of detection, and the MULTI-ARRAY system had the most linear signal output over the widest concentration range (10⁵ to 10⁶). Cytokine concentrations in unspiked and cytokine-spiked serum samples from healthy individuals were further investigated with the MULTI-ARRAY and Bio-Plex assays.

CONCLUSIONS: The MULTI-ARRAY and Bio-Plex multiplex immunoassay systems are the most suitable for biomarker analysis or quantification.

Clinical proteomic research is directed toward the identification of biomarkers that can aid in diagnosing disease, estimating prognosis, and monitoring treatment (1, 2). Large numbers of candidate biomarkers are typically identified in the discovery phase of a proteomic screen, and many candidate biomarkers have been reported in the literature (3). These candidates must be further evaluated to identify those markers that have a statistically significant association with disease (4, 5). In the biomarker verification/validation phase, the concentrations of candidate proteins must be measured across clinical cohorts (6, 7).

The concentrations of circulating proteins in plasma or serum can be measured with ELISAs (8). Although ELISAs provide precise and accurate results, they measure only a single antigen. Recent advances in mass spectroscopy-based protein identification have generated an avalanche of candidate biomarkers, thereby creating a market for high-throughput multiplex immunoassays that allow simultaneous quantification of many analytes. Two basic assay formats have been developed to facilitate simultaneous quantification of multiple antigens: planar array assays and microbead assays. In the first format, different capture antibodies are spotted at defined positions on a 2-dimensional array. In the second, the capture antibodies are conjugated to different populations of microbeads, which can be distinguished by their fluorescence intensity in a flow cytometer. In this study, we evaluated the performance of 3 planar array assays [MULTI-ARRAY (Meso Scale Discovery), A² (Beckman Coulter), and FAST Quant (Whatman Schleicher & Schuell BioScience)] and 2 microbead assays [Bio-Plex (Bio-Rad Laboratories) and FlowCytomix (Bender MedSystems)]. For details about the various platform characteristics, see the Data Supplement that accompanies the online version of this Brief Communication at <http://www.clinchem.org/content/vol56/issue2>.

The accuracy of quantification for multiplexed immunoassays depends, as with all ELISAs, on the quality of the calibration curves, which is determined by the following: appropriate curve-fitting procedures, assay imprecision (CV), recoveries, and assay linearity (the limits of quantification). The performance characteristics of all the platforms were evaluated with respect to these parameters. For each assay system, duplicate or triplicate wells of supplier-provided calibrators were serially diluted as recommended by the manufacturer to produce calibration curves. We were unable to investigate cross-reactivity between analytes because the manufacturers provided the calibrators as premixed solutions containing all of the analytes.

It is important to note that none of the cytokine assays on the 5 platforms have been cleared by the US

Food and Drug Administration for clinical testing. They are to be used only in research applications.

The FlowCytomix assay was not further evaluated as a platform for clinical validation, owing to substantial losses of beads that might adversely affect performance. In this assay, multiplexing is achieved by coupling the detector antibodies to internally fluorescent beads of 2 different sizes (4.4 μm and 5.5 μm); however, we experienced substantial losses of the small beads when we assayed human serum samples. The loss of the small beads may have been due to the viscosity of the serum because, in contrast to the other kits, the manufacturer of the FlowCytomix kit did not supply a diluent specifically formulated for serum.

Most of the currently available multiplex immunoassays have been designed to quantify the concentrations of various cytokines, because cytokine concentrations can provide information about numerous diseases and inflammatory conditions (9, 10). Therefore, performance was analyzed for the remaining platforms, and the results were compared with data obtained for serial dilutions of 5 common cytokines [Fig. 1 for interleukin-6 (IL-6)⁴; Fig. 1 in the online Data Supplement for data for IL-12p70, IL-2, IL-10, and IL-1 β]. All assays were performed on at least 3 different days.

The MULTI-ARRAY system had the greatest linear signal output, over the widest range (10^3 to 10^6), for every cytokine. By comparison, the signal output range was 10^3 to 10^4 for the Bio-Plex assay, 10^3 for the A² assay, and 10^4 for the FAST Quant assay (Fig. 1A and Table 1; Fig. 1A in the online Data Supplement). Within each batch, CVs varied broadly, depending on the analytes: 0.4%–23% for the MULTI-ARRAY assay, 0.6%–40% for the Bio-Plex assay, 0.5%–39% for the A² assay, and 0.8%–13% for the FAST Quant assay (Fig. 1B; Fig. 1B in the online Data Supplement).

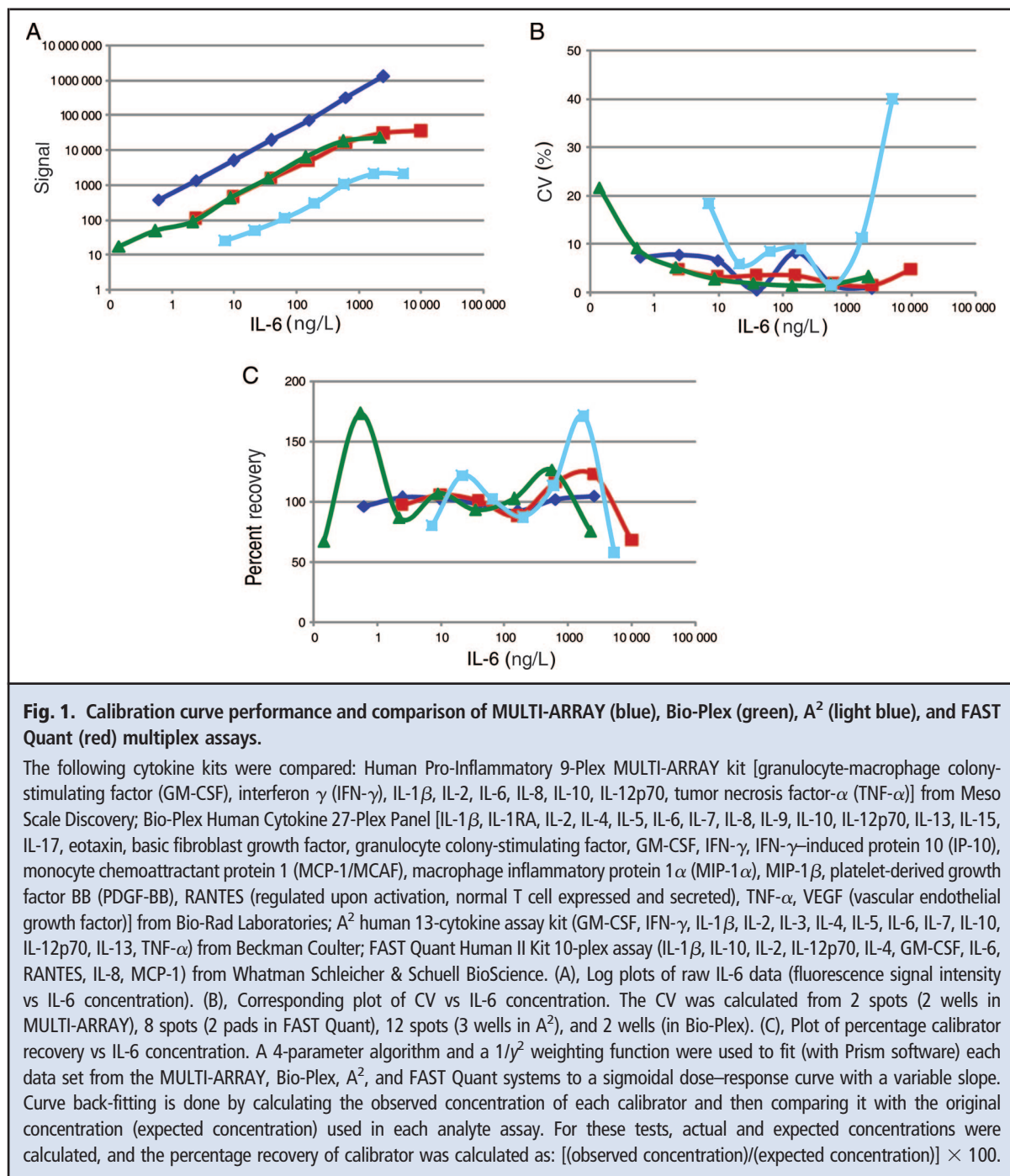
Because cytokines are present at low concentrations in nonpathologic sera, curve fitting has a direct effect on the accuracy of the results. Although each assay system includes software for curve fitting by nonlinear regression, differences among the algorithms and parameters embedded in these proprietary software programs made it impossible to compare the 4 different systems directly with regard to the quality of the results. To standardize the analytical process, we reanalyzed the data with Prism software (GraphPad Software). We used a 4-parameter algorithm and a $1/y^2$ weighting function to fit each data set to a sigmoidal dose–response curve with a variable slope.

To assess the quality of the fit at each concentration, we determined and compared actual and expected values to calculate percentage recovery (Fig. 1C; Fig. 1C in the online Data Supplement). Percentage recovery is an important measure for evaluating the accuracy of quantitative data from the lower limit of detection across the entire dynamic range of an assay (Fig. 1C; Fig. 1C in the online Data Supplement). We defined an upper limit of quantification (ULOQ) as the value below which the percentage recovery was 80%–120% and the CV was <25%. The lower limit of quantification (LLOQ) was defined as an analyte concentration that has (a) a response at least 3 times that of a blank sample, (b) a percentage recovery of 80%–120%, and (c) a CV <25%. We then calculated ULOQ and LLOQ values for the 5 cytokines on each assay platform (Table 1). Overall, the MULTI-ARRAY platform from Meso Scale Discovery had the largest quantifiable concentration interval (2.4–2500 ng/L).

Our assessments identified deficiencies in the A² and FAST Quant platforms that may limit their overall suitability. The LLOQ was relatively poor on the A² platform; similarly, the FAST Quant assay was not as sensitive as the other assays (Table 1). The FAST Quant software uses a point-to-point curve-fitting method and does not provide data on the back-fitting of a calibration curve (percentage recovery), making it difficult to evaluate the accuracy of the data.

The MULTI-ARRAY and Bio-Plex platforms demonstrated the best performance for quantifying purified cytokines. Although both systems were highly sensitive, the MULTI-ARRAY had a wider dynamic range because it had a higher ULOQ. On the other hand, the Bio-Plex system was able to quantify more analytes. We tested the ability of the MULTI-ARRAY and Bio-Plex systems to measure a common set of 9 cytokines in the complex milieu of human serum. Human serum samples from 12 healthy male individuals between 20 and 60 years of age were purchased from Bioreclamation. As expected, the concentrations of most cytokines in these healthy controls were very low, with many of the readings below the LLOQ (see Table 1 in the online Data Supplement). For samples of unspiked serum, the MULTI-ARRAY platform had better performance for 6 cytokines (IL-2, IL-10, IL-12p70, granulocyte-macrophage colony-stimulating factor, interferon γ , and tumor necrosis factor- α), and the Bio-Plex system had a better performance for 1 cytokine (IL-8). The 2 platforms performed similarly for IL-6. IL-1 β could not be quantified with either platform. The inter- and intraplate CVs for duplicate assays were low for both platforms (mean interplate CVs of 8% and 10% for the MULTI-ARRAY and Bio-Plex

⁴ Nonstandard abbreviations: IL-6, interleukin-6; ULOQ, upper limit of quantification; LLOQ, lower limit of quantification.



platforms, respectively; mean intraplate CVs of 6% and 9% for the MULTI-ARRAY and Bio-Plex platforms, respectively).

To investigate the accuracy of cytokine quantification in human serum, we measured cytokine concentrations in serum samples supplemented with pure cytokines (see Fig. 2 in the online Data Supplement).

Because we added each cytokine to a concentration of 50 ng/L, the difference between the measured cytokine concentrations in the supplemented and unspiked samples should have been 50 ng/L. On the MULTI-ARRAY platform, the differences varied from 53 ng/L to 70 ng/L. The Bio-Plex platform had larger differences (15–78 ng/L). One limitation of this study is that

Table 1. Comparison of the performance of 4 multiplex immunoassay platforms.^a

	MULTI-ARRAY	Bio-Plex	A ²	FAST Quant
IL-12p70				
Manufacturer's calibrator interval, ng/L ^b	2500–0.6	4411–0.3	4800–6.5	50 000–12.2
Quantifiable interval, ng/L ^c	2500–0.6	4411–0.27	577–7.1	625–2.4
Signal interval ^d	≈2 700 000–200	≈23 000–30	≈1000–25	≈16 000–100
Mean CV within quantifiable interval ^e	9.60%	2.80%	8.70%	3.40%
IL-2				
Manufacturer's calibrator interval, ng/L	2500–0.6	2161–0.1	6630–9.1	10 000–2.4
Quantifiable interval, ng/L	2500–2.4	540–2.1	245–9	10 000–2.4
Signal interval	≈600 000–200	≈18 000–100	≈4000–100	≈2000–30
Mean CV within quantifiable interval	5.00%	5.90%	10.00%	3.20%
IL-6				
Manufacturer's calibrator interval, ng/L	2500–0.6	2215–0.1	5200–7.1	10 000–2.4
Quantifiable interval, ng/L	2500–0.6	138–2.1	577–7.1	625–2.4
Signal interval	≈1 300 000–400	≈6000–80	≈1000–25	≈16 000–100
Mean CV within quantifiable interval	4.70%	2.80%	8.70%	3.40%
IL-10				
Manufacturer's calibrator interval, ng/L	2500–0.6	4311–0.3	4750–6.5	50 000–12.2
Quantifiable interval, ng/L	2500–0.6	269–1.05	175–6.5	50 000–195
Signal interval	≈500 000–300	≈70 000–20	≈3000–70	≈1500–300
Mean CV within quantifiable interval	5%	8%	6%	4%
IL-1β				
Manufacturer's calibrator interval, ng/L	2500–0.6	3794–0.2	4840–6.6	10 000–2.4
Quantifiable interval, ng/L	2500–0.6	59–0.2	538–6.6	625–2.4
Signal interval	≈1 300 000–500	≈9000–50	≈3000–50	≈20 000–400
Mean CV within quantifiable interval	6.60%	5.30%	8.40%	5.00%
Calibrators				
Manufacturer's recommended standard dilution factor ^f	1/4 Dilution	1/4 Dilution	1/3 Dilution	1/4 Dilution
Calibration curve	7 Concentrations	8 Concentrations	7 Concentrations	7 Concentrations

^a The manufacturers of the different multiplex platforms are Meso Scale Discovery (MULTI-ARRAY), Bio-Rad Laboratories (Bio-Plex), Beckman Coulter (A²), and Whatman Schleicher & Schuell (FAST Quant).

^b With the manufacturer's recommended serial dilution of calibrators (see manufacturer's recommended standard dilution factor).

^c The quantifiable interval of each analyte is defined as the concentration interval in which the CV is <25% and the percentage recovery is <100% ± 20% with the standard calibrators.

^d The signal interval of each analyte is defined as the signal output between upper and lower quantification limits with a CV <25% and a percentage recovery <100% ± 20% with the standard calibrators.

^e The mean CV within the quantifiable interval is calculated with a CV of <25% and a percentage recovery <100% ± 20% with the standard calibrators.

^f Serial dilution factors are expressed as the fraction of the concentration before dilution.

we did not evaluate potential effects related to pathophysiological changes in serum composition or the molecular form of the analytes. Thus, this study presents the best possible performance of the assays, and performance with real-world samples could be substantially worse.

The performance of multiplex assay platforms is analyte, sample type (matrix), and concentration dependent. Optimal assay performance depends on proprietary information about the antibody pair, the composition of diluents, and the software. Therefore, no

single assay platform is suitable for all analytes. The MULTI-ARRAY and the Luminex-based Bio-Plex assays performed best overall, but their performance characteristics were not uniform (see Table 1 in the online Data Supplement). The 2 platforms performed similarly in detecting high concentrations (50 ng/L) of spiked cytokines (see Fig. 2 in the online Data Supplement). Although the MULTI-ARRAY platform was superior for quantifying cytokines in unspiked serum, it was severely limited by the small number of analytes that can be assayed with commercially available kits,

which are provided exclusively by Meso Scale Discovery. In contrast, assays for a large selection of analytes are available from a network of Luminex partners. Luminex assays also have the potential to multiplex up to 100 analytes, whereas MULTI-ARRAY assays are limited to 10-plex.

In summary, the simultaneous analysis of multiple antigens makes it possible to identify combinations of biomarkers that have greater disease specificity and sensitivity than results obtained from the analysis of any single marker (11–15). We compared 5 commercial multiplex immunoassay platforms (representing both planar array and microbead assays) and found the MULTI-ARRAY and Bio-Plex systems to be the most suitable for biomarker verification and validation; however, the performance of even these systems was analyte dependent. Our results highlight the importance of an assay's dynamic range, linearity, CV, and percentage recovery in obtaining accurate and reproducible assay results.

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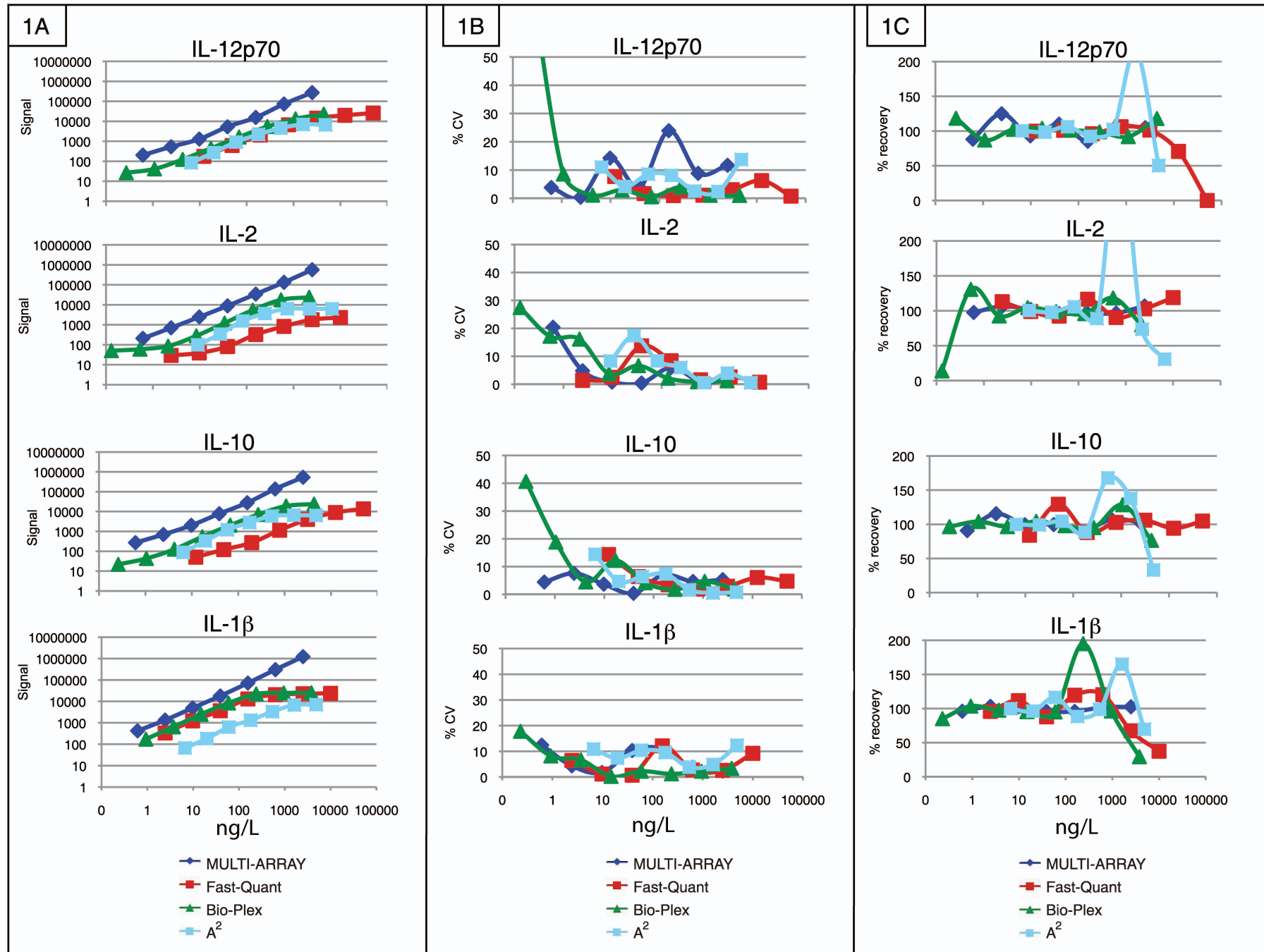
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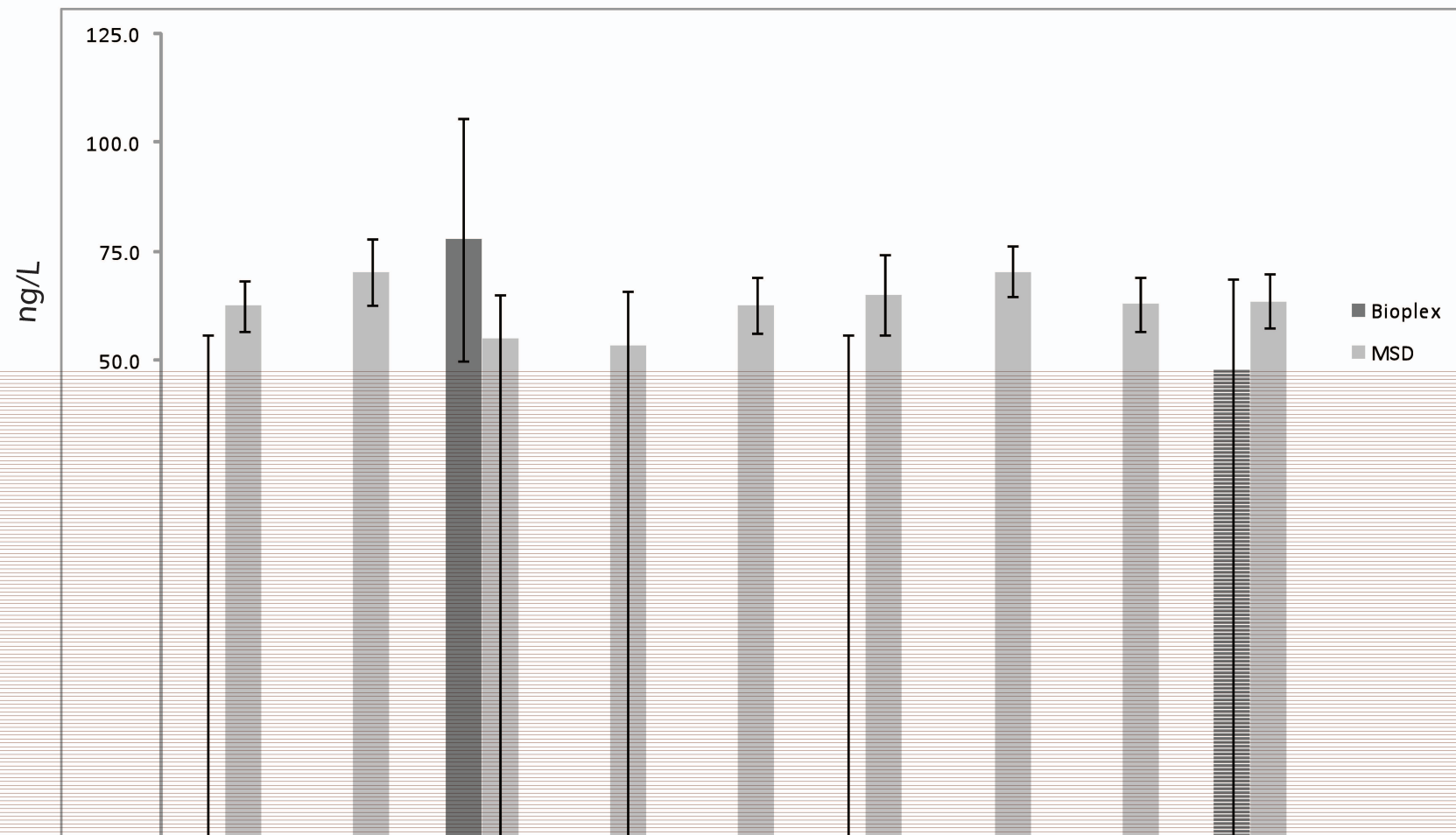
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Supplementary Figure 1.

Supplementary figure 2



Supplement Table I

Platform	Cytokine	Quantifiable range (ng/L) ¹	# samples in 12 control serum >LLOQ ²	concentration range (neat serum >LLOQ) (ng/L) ³	intra-plate concentration CV ⁴	inter-plate concentration CV ⁵
MULTI-ARRAY	IL-1b	25000.6	1/ 12	0.7	6%	7%
	IL-2	2500-0.6	8/ 12	0.6-4.3	6%	8%
	IL-6	25000.6	12/ 12	0.96.1	7%	7%
	IL-8	2500-0.6	12/ 12	2.8-195	6%	12%
	IL-10	2500-0.6	12/ 12	0.9-14.5	10%	9%
	IL-12(p70)	2500-2.4	5/ 12	1.4-23.2	5%	8%
	GM-CSF	2500-0.6	8/ 12	0.8-2.2	7%	9%
	IFN-γ	25002.4	9/ 12	2.49.7	4%	9%
	TNF-α	2500-0.6	12/ 12	3.2-14.5	7%	9%
Bio-Plex	IL-1b	600.60.2	0/ 12	<LLOQ	2%	8%
	IL-2	5439-1.3	3/ 12	2.9-17.2	21%	21%
	IL-6	80282.4	12/ 12	2.57.2	5%	8%
	IL-8	6613-0.4	11/ 12	2.3-84.1	11%	9%
	IL-10	1758-0.1	7/ 12	0.3-0.7	0%	8%
	IL-12(p70)	96300.6	0/ 12	<LLOQ	5%	5%
	GM-CSF	11547-2.8	0/ 12	<LLOQ	12%	14%
	IFN-γ	66137.8	1/ 12	27.9	8%	8%
	TNF-α	22533-1.4	1/ 12	6.7	11%	8%

¹ Quantifiable range of each analyte is defined by a concentration range where CV was <25% and % recovery was <100+/- 20% from standard calibrators. For LLOQ, the signal was at least 3 x above the blank wells.

² # of samples > LLOQ represents the number of control serum samples out of 12 samples (out of 12 samples) were measured and calculated to be above LLOQ for specified cytokines.

³ Specified cytokine concentration range represents the control samples (out of 12 samples) which neat concentration are > LLOQ. If all 12 serum samples' concentration < LLOQ for specified cytokines are labeled as <LLOQ.

⁴ CV was calculated from the value obtained from the two aliquots of the same spiked sample measured on the same plate in duplicate.

⁵ CV was calculated from the value obtained from the two aliquots of the same spiked sample measured on the two difference plates in duplicate.

Supplement materials:

Multiplex platform characteristics

In the MUTI-ARRAY, from Meso Scale Discovery (MSD), capture antibodies are spotted on carbon surfaces at the bottoms of the wells of a MULTI-SPOT plate. Antigen levels are quantified using specific detector antibodies labeled with a SULFO-TAGTM, which emits light upon electrical stimulation. In the A² protein microarray system, from Beckman Coulter, capture antibodies are conjugated to specific oligonucleotides and then bound to complementary oligonucleotides which are microspotted in the wells of a 96-well plate. Antigens are detected with a biotinylated detector antibody and a streptavidin-conjugated fluorophore. In the Fast Quant multiplex cytokine quantification system, from Whatman Schleicher & Schuell BioScience, capture antibodies are spotted on 16-pad FAST Slides, which have a nitrocellulose surface. Biotinylated detector antibodies and streptavidin-Cy5 are used for quantification. In the Bio-Plex suspension array, from Bio-Rad, capture antibodies are coupled to a family of fluorescently-tagged beads developed by Luminex (Luminex's xMAP technology). A flow cytometer with two lasers can distinguish between different beads based on their fluorescence intensity, and then quantify the amount of a fluorescent reporter (phycoerythrin or PE) coupled to the detector antibody. The FlowCytomix system, from Bender MedSystems, employs capture antibodies coupled to fluorescent polystyrol beads of different sizes. A dual-laser flow cytometer can identify the beads based upon their size and fluorescence intensity, and then quantify the amount of antigen by measuring fluorescence emitted from Streptavidin-Phycoerythrin (PE) associated with a biotinylated detector antibody.

Method

Re-analyzing the data with PRISM software

In addition to the missing information regarding to the percent recovery, the output result (pg/ml) from Fast Quant software is an average of 3 spots, and the software excluded spots with signal intensities less than the background (e.g. diluents-only spots) which can result in the “averaged reported data” being from less than three spots and thereby reducing the reported CV%. The results could be misleading. For one sample, there were three signals with values of 0, 442, and 19. The final output of 619 ng/L from the FastQuant software was based on only the highest signal (442) because the spots with lower signals were excluded from the calculation because their values were below the background from the diluents. The reported CV of 0% was inaccurate because it was based on only one spot out of three replicated spots.

Using GraphPad software PRISM software (GraphPad, La Jolla, CA) to re-process the raw data, the LLOQs (based on our definition of recovery between 80-120%, and CV< 25%) in general were higher (indicating a less sensitive assay) than the values reported by the manufacturers. As well, we were able to flag samples where the calculated concentrations were less than the LLOQ of neat serum samples (supplement table I), which should reduce the false positive rate.

Supplement figure legend:

Supplement Figure 1 : Standard curve performance and comparison of MULTI-ARRAY, Fast Quant, Bio-Plex and A². The following cytokine kits were compared: Human pro-inflammatory (9-Plex)

MULTI-ARRAY kit from Meso Scale Discovery; Bio-Plex Human Cytokine 27-Plex panel from Bio-Rad Laboratories; A² human 13 cytokine assay kit from Beckman Coulter; Fast Quant Human II 10-plex from Watman Schleicher & Schuell BioScience; and FlowCytomix human TH1/Th2 10-plex kit from Bender Medsystem. Panel A shows the raw data log plotting fluorescent signal intensity versus concentration of IL-12p70, IL-2, IL-4, IL-10 and IL-1b. The corresponding coefficient of variation (CV) versus concentration is presented in panel B. The % CV was calculated from two spots (two wells in MULTI-ARRAY), eight spots (two pads in Fast Quant), 12 spots (three wells in A²) and two wells in Bio-Plex. Panel C shows standard recovery versus concentration. Each data set from MULTI-ARRAY, Fast Quant, Bio-Plex, and A² was fit (utilizing PRISM software from GraphPad) to a sigmoidal dose-response curve with a variable slope, using a four-parameter algorithm and a $1/y^2$ weighting function. Curve back-fitting is done by calculating the actual, observed concentration of each standard, and then comparing it to the original concentration (expected concentration) used in each analyte/assay. For these tests, actual and expected concentrations were calculated, and the standard, or percent, recovery (observed concentration / expected concentration X 100) is presented in 1C.

Supplement Figure 2: Measurement of 50 pg/ml cytokine in the context of control human serum samples. Nine cytokines were quantified on the MULTI-ARRAY and Bio-Plex platforms in neat serum samples and in serum samples spiked with a mixture of 50 ng/L of each cytokine. The data presented represent the difference between the two readings (N=12, error bars = standard deviation). If the reading for neat serum was below the LLOQ, 0 ng/L was subtracted. The assay was done with duplicate wells. A 8 point standard standard curve was established for MULTI-ARRAY and a 11 point standard curve was established for Bio-Plex. Concentrations were calculated by utilizing PRISM software from GraphPad to a sigmoidal dose-response curve with a variable slope, using a four-parameter algorithm and a $1/y^2$ weighting function.