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Development, validation, and implementation of a multiplex immunoassay for the simultaneous determination of five cytokines in human serum

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Abstract

Quantification of biomarkers can provide important information about the safety and efficacy of candidate drugs. Unfortunately, limited sample volume and excess costs often limit analysis of multiple biomarkers. We developed, optimized, validated, and implemented a multiplex immunoassay for simultaneous measurement of multiple circulating cytokines: IL-1β, TNFα, IL-6, IL-8, and IL-10.

Multiplex immuoassays were performed using the Luminex LabMAP instrument. Capture antibodies for each cytokine were covalently bound to distinct microsphere subsets distinguished by differing dye ratios. The concentration of each individual cytokine determined by measuring orange fluorescence produced by a complex of a biotinylated cytokine-specific antibody and streptavidin-phycoerythrin.

The lower limit of quantification for all assays was 20 pg/mL with the exception of IL-8 which was 100 pg/mL. The inter-assay precision was less than 25%CV for all analytes at all control levels both pre-study and in-study. The percent recovery ranged from 83 to 108% pre-study and 90 to 125% in-study. In a linearity assessment, a 15,000 pg/mL multi-analyte control could be diluted 1:50 and maintain expected accuracy.

We measured the cytokine concentrations in more than 2000 serum samples from patients with sepsis. Multiplex results for IL-6 were compared to a conventional commercially available ELISA kit. The degree of agreement between the two methods as measured by the concordance correlation coefficient was 84.5%. Multiplex results were 2.36-fold higher than ELISA values on the average. After adjusting for this mean difference, the 95% empirical limits of agreement for the ratio of individual sample values were 0.33, 2.65.

This multiplex immunoassay provided simultaneous measurement of circulating cytokines using 80% less patient specimen compared to traditional approaches and at a significantly decreased cost. Efficient use of this platform requires process improvements to fully maximize the positive impact of multiplex assays in clinical drug development.

Keywords: Multiplex; LabMAP; Microsphere; Fluorescence; Immunoassay; Cytokines; Analytical validation

1. Introduction

Cytokines play important roles in diverse physiological functions, including inflammation, cell differentiation, promoting chemotaxis, and generating an acute phase response [1]. Sepsis is one example where changes in multiple cytokines mediate the disease pathogenesis [2]. Specifically, pro-inflammatory cytokines (IL-1β, TNFα, IL-8, and IL-6) have been implicated in the pathogenesis of sepsis [3–5]. Redundancy, interdependence, and antagonism of the in vivo cytokine network often confound the interpretation of data from the determination of a single cytokine. A better approach for investigating putative relationships between cytokine production and disease states is to simultaneously profile temporal changes in multiple cytokines. While more informative, the measurement of multiple cytokines during clinical drug studies is laborious, time-consuming, costly and places greater demand on the collection volume for

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whole blood. Therefore, an effort was undertaken to develop and validate a multiplex fluorescent sandwich immunoassay using the Luminex platform to measure multiple cytokines in serum of patients with sepsis, simultaneously.

Luminex technology has been applied successfully for measurement of various biomarkers, including cytokines, in biological matrices [6–8]. Accordingly, we undertook the current study to investigate the suitability of Luminex technology for Good Clinical Practice-compliant quantification of multiple cytokines in support of clinical trials for new investigational drugs. Unlike assays of conventional drugs for pharmacokinetic and bioequivalency assessments [9,10], formal regulatory guidance is lacking for validation of biomarker assays. Therefore, we relied on existing guidance documents and recent publications for validation of assays to support pharmacokinetic assessments of macromolecular therapeutics [11,12] to provide a framework for designing the validation of a multiplexing assay for cytokines.

The multiplex method simultaneously measures concentrations of five cytokines (IL-1β, TNFα, IL-8, IL-10 and IL-6). The assay was originally developed and optimized at Eli Lilly and Company, and the scale-up and manufacturing process was conducted in a GMP manufacturing facility. All results shared in this manuscript were generated from the GMP quality kits. A pre-study validation characterized analytical sensitivity, specificity, accuracy, precision, linearity, and freeze–thaw stability. The in-study analysis evaluated analytical accuracy and precision based on daily analysis of quality control samples over the duration of the study. All five cytokines were measured in 2086 sera samples from sepsis patients. The IL-6 concentrations measured by the multiplex assay were compared to IL-6 levels determined by a single analyte ELISA in an independent laboratory.

2. Experimental

2.1. Materials and equipment

All standards and antibodies were purchased from R&D Systems (Minneapolis, MN). Standards were recombinant human IL-1β (201-LB-005), recombinant human IL-6 (206-IL-010), recombinant human IL-8 (208-IL-010), and recombinant human TNFα (210-TA-010). The capture antibodies were mouse monoclonal (Human IL-1β, MAB601; Human IL-6, MAB206; Human IL-8, MAB206; and Human TNFα, MAB610). The secondary antibodies used for detection of the binding reaction were biotinylated goat anti-human polyclonals (Human IL-1β, BAF201; Human IL-6, BAF206; Human IL-8, BAF208; and Human TNFα, BAF210). Linco Laboratories Inc. provided all IL-10 reagents and the specifications were not provided. Streptavidin labeled phycoerythrin (S-866) was purchased from Molecular Probes (Eugene, OR). Heat-treated charcoal stripped serum was obtained from Western States Plasma (Oceanside, CA). Normal mouse serum was purchased from Jackson ImmunoResearch Inc. (West Grove, PA). [1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrohloride] (EDC) was purchased from Pierce (Rockford, IL). The 96-well multiscreen (MABVN1250) filter plates were from Millipore (Bedford, MA). Titer plate shaker was purchased from Lab Line Instruments Inc. (Melrose Park, IL). Both fluorescently labeled microspheres and a Luminex 100 were obtained from Luminex Corporation (Austin, TX).

2.2. Microsphere conjugation

The antibodies were covalently linked to the surface of the fluorescently labeled microspheres by a two-step procedure using EDC [13]. The antibodies were added at 230 μg/mL to 5 million beads and rotated for 2 h.

2.3. Standard curve preparation

Standards were provided as lyophilized cytokine cocktails (15,000 pg/mL). Standards were prepared fresh daily by adding 1 mL of heat-treated charcoal stripped serum. Each analyte had the same concentration values ranging from 6.2 to 15,000 pg/mL. The individual standards were prepared by performing half-log serial dilutions.

2.4. Immunoassay reagents

All solutions were stored at 4 °C. Assay buffer was composed of 50 mM HEPES, 150 mM NaCl, 0.05% Tween 20, 0.1% BSA, 0.1% sodium azide, 1% EDTA, 0.2% mouse serum, pH 7.5. Blocking buffer consisted of 50 mM HEPES, 2% HSA, 0.1% sodium azide, pH 7.5. Wash buffer was composed of (Dulbecco's PBS, 0.02% Tween 20, 0.1% BSA, 0.05% sodium azide, pH 7.4.

2.5. Development of a multiplex immunoassay using Luminex technology

The immunoassay procedure was carried out using a liquid handling device (Tecan Genesis 150, Hombrechtikon, Switzerland). The procedure was conducted as follows: 96-well filter plates were blocked by adding blocking buffer 150 μL/well for 30 min at room temperature. After aspirating the liquid phase, 100 μL of each specific bead set was added to each well for a total of 5000 beads per set. The buffer was removed by vacuum filtration. Samples and standards prepared in heat-treated charcoal stripped serum were diluted 1:2.5 with assay buffer. After dilution, 100 μL additions of samples or standards were added to the plate and incubated overnight (16–24 h) at 4 °C with gentle shaking on a plate shaker. Following the incubation, the liquid phase was removed by vacuum filtration. The plate was washed four additional times by adding 200 μL of wash buffer. Following the final aspiration,

100 μL of a cocktail of biotinylated secondary antibodies [IL-1β (0.5 μg/mL), TNFα, IL-6 (3.0 μg/mL), and IL-8 (0.375 μg/mL)] were added to each well. The secondary antibodies were incubated at room temperature with maximum shaking on a titer plate shaker. After 1 h, the plate was vacuumed and washed four times again with 200 μL of wash buffer. After the final aspiration, 100 μL of 5 μg/mL streptavidin-PE diluted in Dulbecco's-PBS, pH 7.4 was added to each well. The streptavidin-PE was incubated for 15 min at room temperature with maximum shaking on a titer plate shaker. After removing the liquid phase, 100 μL of 4.0% formaldehyde diluted in water was added to each well. The 96 well plate was placed in the XY platform of the Luminex 100. From each well a minimum of 50 analyte specific beads were analyzed for both bead designation and the phycoerythrin fluorescence. For the standards, phycoerythrin values were plotted against standard concentrations, and the data were fit with a weighted 4/5-parameter logistic model (StatLIA, Brendan Scientific, Grosse Pointe, MI) [14]. The quality control samples and unknown concentrations were deduced from the standard curve.

2.6. Bulk reagent production

Bulk reagents were 'scaled up' and manufactured in accordance with good manufacturing practices (GMP) by (Linco Research Laboratories, St. Charles, MO). From this point, the experiments described were generated using kits manufactured by (LINCO Research Laboratories, St. Charles, MO). IL-10 reagents were proprietary. Bulk reagents were provided in kits with individually packaged microspheres, lyophilized standards and controls, secondary antibody and reporter diluted to the appropriate concentration. Other accessory components included a filter plate and adhesive covers. All light sensitive reagents were placed in light protected bottles to maintain fluorescent properties, and the reagents were contained in a hard plastic light protective case.

2.7. Analytical validation: sensitivity and precision

Analytical performance of the multiplex immunoassay was defined in a comprehensive pre-study validation. The guidelines described by Findlay et al. [11] were applied for pre-study validation. Validation samples were prepared by adding cytokines to heat-treated charcoal stripped serum (HTCSS) at 20, 50, 100, and 3000 pg/mL. The validation samples were assayed a total of 48 times over the course of 3 days, employed 12 plates, and two operators. The a priori criteria required that percentage of recovery values must be within 20% of nominal, and the maximum inter-assay precision must be <25% [11]. Precision and accuracy were determined using a previously described method [11].

2.8. Antibody specificity and assessment of high dose hook effect

Assay specificity was evaluated by adding a single analyte at a concentration of 1000 pg/mL to a well containing all five microsphere sets. The assay for each cytokine was deemed specific only if the concentrations of the analytes were below the quantifiable limit of 20 pg/mL except the single analyte added to the well. A high dose hook effect was evaluated by addition of 100,000 pg/mL of a single analyte assayed with all five microsphere sets. A hook effect was determined by an interpolated value in the reportable range (20–3000 pg/mL).

2.9. Dilutional linearity

Dilutional linearity was assessed by diluting a 15,000 pg/mL cytokine cocktail prepared in heat-treated charcoal stripped serum. The sample was diluted 1:5, 1:10, 1:25, and 1:50 with heat-treated charcoal stripped serum. The results were calculated from the standard curve and the concentration was multiplied by the dilution factor. The pre-defined criteria for acceptable linearity were ±20% recovery and <20%CV of the replicates.

2.10. Analyte freeze–thaw stability

A 3000 pg/mL multi-analyte control was reconstituted with human serum from a healthy subject. The samples were split into four groups corresponding to the freeze/thaw cycle. The control was spiked, frozen and stored at −70 °C, thawed, and assayed on the same day as the other freeze–thaw samples. For each sample, a total of four values were obtained and the standard deviation and the mean were computed. The a priori criteria stated that freeze–thaw samples could not exceed ±20% recovery compared to the control.

2.11. In-study assessment of accuracy and precision

During the analysis of patient samples, three levels of quality control samples (50, 100 and 3000 pg/mL) were analyzed. All three concentrations of the quality control samples were analyzed three times during an analytical run in different positions on the plate. The one exception was IL-8. This particular analyte had only two levels of quality control values (100 and 3000 pg/mL) because the low-end sensitivity was not sufficient to measure at concentrations <100 pg/mL. For each analyte, the accuracy and precision of an analytical run was judged to be acceptable if two-thirds of all quality control values were within 35% of the nominal values, with at least one result within 35% for each control.

2.12. Comparison between ELISA and Luminex multiplex

The IL-6 results obtained by the Luminex-based method above were compared with results for the same analyte

Table 1
Pre-study accuracy assessment (%relative error)

Analyte	Nominal concentration			
	20 pg/mL	50 pg/mL	100 pg/mL	3000 pg/mL
IL-6	−1.0	−10.8	−13.3	−7.7
IL-8	31.3[a]	3.2	−4.7	−16.3
IL-10	−2.6	−11.0	−11.6	−9.6
IL-1β	7.8	0.9	2.1	−5.7
TNFα	8.8	4.9	4.8	−2.8
Replicates	48	48	48	36

[a] Represents a value outside the a priori criteria. The results were collected over 3 days, 12 plates, and two analysts.

determined using a High-Sensitivity ELISA kit (R&D Systems, Minneapolis, MN, catalog # HS600).

3. Results

3.1. Pre-study assessment of accuracy and precision

Accuracy and precision results from 3 days of analysis and 12 plates are summarized in Tables 1 and 2, respectively. Recovery values are expressed as a percent relative error (%RE) and inter-assay precision values are expressed as a percent coefficient of variation (%CV). All analytes except IL-8 at 20 pg/mL met the a priori accuracy criteria of ±20% of nominal. Similarly, inter-assay precision values (Table 2) for all analytes except IL-8 at 20 and 50 pg/mL satisfied the a priori precision criteria of <25%CV. Based on these results, the limits of quantification were established as 20–3000 pg/mL for all analytes except IL-8 for which the limits were truncated as 100–3000 pg/mL.

3.2. Selectivity/specificity

Since all five cytokines are measured simultaneously, analytical specificity for each is imperative. Specificity was determined by adding all five microsphere sets with 1000 pg/mL of a single analyte. The antibodies were deemed cross-reactive if a value of >20 pg/mL was obtained. No cross-reactivity occurred with these antibodies. The potential for a high dose hook effect was assessed by adding all five bead sets with 100,000 pg/mL of a single analyte. None of the antibodies demonstrated a hook effect. In addition to selectivity and specificity, the antibodies must be combinable or capable of producing effective standard curves. Fig. 1 represents reference standard curves for each assay superimposed on the same graph.

Table 2
Pre-study inter-assay precision (%coefficient of variation)

Analyte	Nominal concentration			
	20 pg/mL	50 pg/mL	100 pg/mL	3000 pg/mL
IL-6	15.9	11.0	9.1	5.7
IL-8	47.1[a]	28.9[a]	18.4	11.2
IL-10	16.9	15.6	10.7	7.5
IL-1β	22.3	15.5	13.7	7.5
TNFα	17.1	12.0	10.7	9.3
Replicates	48	48	48	36

[a] Represents a value outside the a priori criteria. The results were collected over 3 days, 12 plates, and two operators.

3.3. Dilutional linearity

Dilutional linearity was evaluated to justify sample dilution when analytical results exceed the reportable range. A 15,000 pg/mL cytokine cocktail was diluted 1:5, 1:10, 1:25, and 1:50 with heat-treated charcoal stripped serum. The results were estimated from the calibration curve and the concentration was multiplied by the dilution factor. Recovery of all analytes was ±20% of nominal and the %CV values were <20%. As a result the maximum reportable concentration was determined to be 150,000 pg/mL.

3.4. Freeze–thaw stability

Since some samples required dilution or repeat analysis as a result of a failed run, assessment of the freeze–thaw stability was required. Each analyte was subjected to at least four freeze–thaw cycles, assayed, and compared to a control. Recovery was computed and any value exceeding ±20% of control was determined to be unstable. As shown in Table 3, acceptable recoveries were found for IL-1β and IL-10 through two freeze–thaw cycles, for TNFα and IL-8 through three freeze–thaw cycles, and for IL-6 through four freeze–thaw cycles.

3.5. In-study assessment of accuracy and precision

Daily quality control samples were tabulated from the 71 different plates with three results per plate. A total of 213 results per concentration were collected. Each control level for all five analytes had inter-assay precision values <20% with the exception of IL-8, Table 4. IL-1β and TNFα had elevated mean recoveries for the low and medium controls, but all of the other analytes had mean recoveries between 90 and 110% of nominal, Table 4. The elevated QC values led to an increased number of failed runs for IL-1β and TNFα

Table 3
Freeze–thaw assessment

Freeze–thaw	Percentage of control				
	TNFα	IL-6	IL-10	IL-8	IL-1β
1	100 (2.4)	100 (0.9)	100 (4.0)	100 (6.5)	100 (7.0)
2	98 (4.0)	106 (3.7)	104 (3.6)	94 (4.7)	93 (7.3)
3	90 (4.9)	94 (5.4)	75 (3.3)	88 (5.5)	62 (1.5)
4	76 (3.4)	83 (1.0)	2 (8.9)	85 (6.9)	3 (7.0)

Parentheses represent %coefficient of variation for quadruplicate determination.

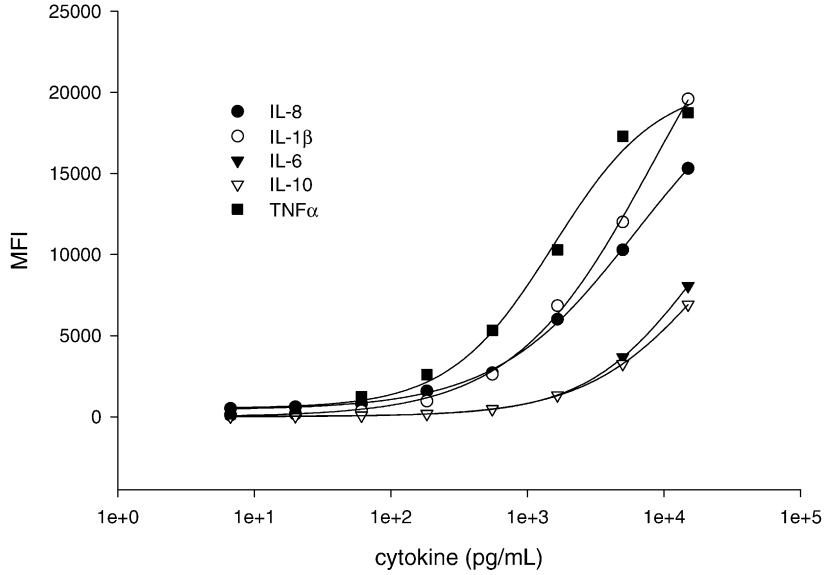

Fig. 1. Representative standard curves for all five analytes and typical standard curves prepared in heat-treated charcoal stripped serum ranging from 6.2 to 15,000 pg/mL. IL-1β, IL-6, IL-8, IL-10, and TNFα were represented by the following symbols: open circles, dark triangles, closed circles, open triangles, and closed squares, respectively.

Table 4
In-study precision (%coefficient of variation) and accuracy (%relative error)

Analyte	Nominal concentration					
	%RE			%CV		
	50 pg/mL	100 pg/mL	3000 pg/mL	50 pg/mL	100 pg/mL	3000 pg/mL
IL-6	9.0	6.7	−3.6	17.7 (14.4)	14.2 (11.2)	11.3 (8.4)
IL-8	NA	13.3	−9.2	NA	24.2 (16.8)	17.2 (14.5)
IL-10	0.5	1.7	−11.2	18.5 (11.9)	13.3 (10.9)	12.3 (8.4)
IL-1β	20.6	24.6	−2.5	16.3 (13.2)	12.5 (9.4)	14.1 (10.0)
TNFα	19.2	19.0	−3.4	12.8 (9.5)	11.3 (8.3)	13.0 (10.4)

Parentheses represent intra-assay variability. Two operators collected the results on 71 plates. The number of replicates available for each of these cytokines was 213.

(11 and 9, respectively). IL-8 had five failed runs and IL-6 and IL-10 had two each.

3.6. Comparison between IL-6 ELISA and IL-6 Luminex assay

A vendor laboratory measured ELISA results for IL-6 using an R&D Systems High-Sensitivity kit, and those corresponding samples were assayed using Luminex technology. Out of 2086 samples assayed, Luminex results from 225 samples were reported to be below the limit of quantification and were therefore excluded from this comparison. The degree of agreement between the two methods for the remaining 1861 samples as measured by Lin's concordance correlation coefficient [15] was 84.5%, Fig. 2a. This correlation indicates that the degree of agreement between the ELISA and Luminex results is 84.5%. Note that unlike the usual Pearson's correlation coefficient which measures the degree of closeness of points to the best straight line, Lin's concordance correlation coefficient measures the degree of closeness of the points to the "agreement line" (ELISA = Luminex line, i.e. the 45° line).

Luminex results were 2.36-fold higher than ELISA results on the average, as measured by the median ratio. After correcting for this systematic bias, the 95% empirical limits of agreement [16] for the ratio of Luminex and ELISA sample values were 0.33, 2.65, Fig. 2b, and Lin's concordance correlation increases to 94.5%.

4. Discussion

Our investigation demonstrated that Luminex technology is a valid platform for simultaneous determination of circulating concentrations of five cytokines. In addition to being analytically valid, the technology was judged to be operationally practical and feasible for application in routine analyses. Luminex multiplexing assays are similar to other

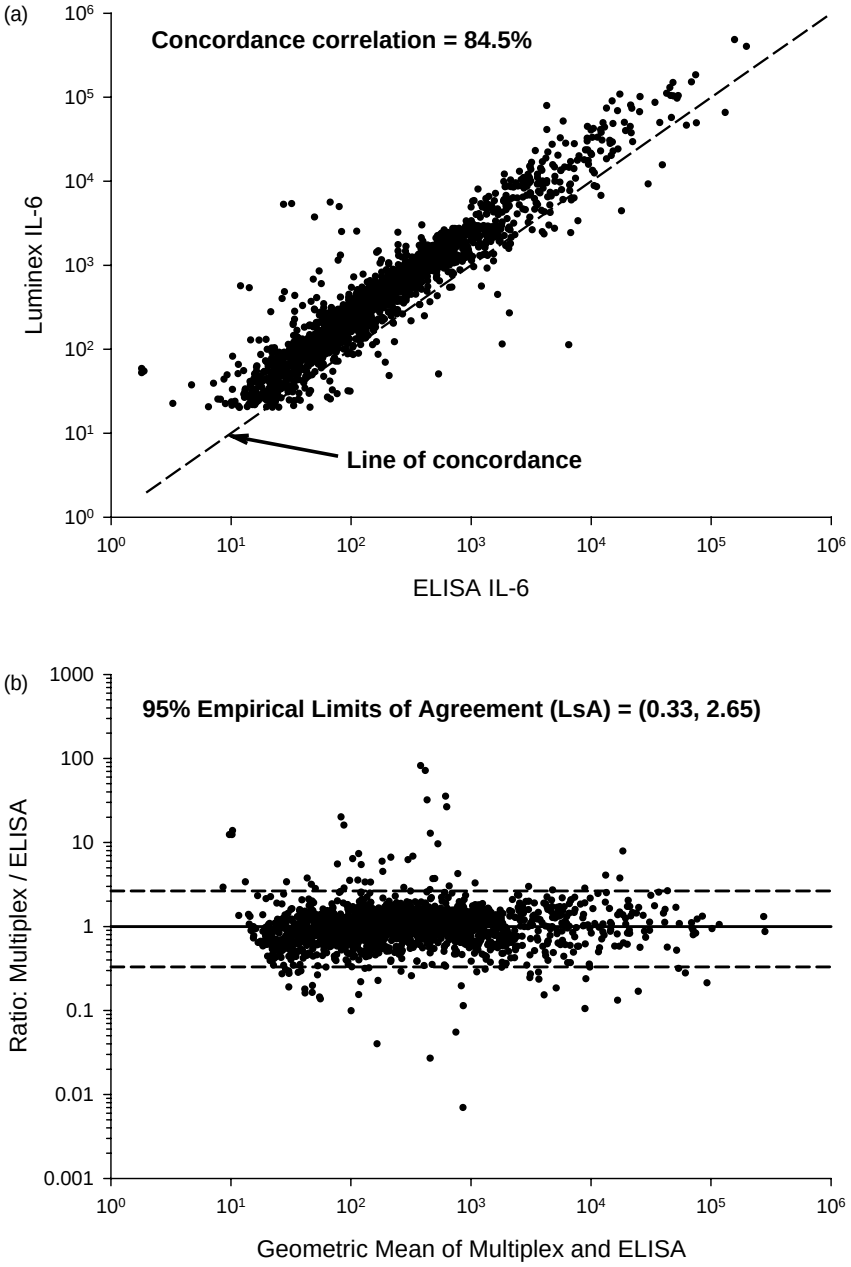

Fig. 2. (a and b) Comparison between Luminex and ELISA IL-6 assays. ELISA results were obtained for 1861 serum samples using a IL-6 R&D Systems High-Sensitivity kit and those corresponding samples were assayed using Luminex technology. In Figure (a), the ELISA data were plotted on the *X*-axis and the multiplex data were plotted on the *Y*-axis titled Luminex. The dash-line denotes the line of agreement between these two methods and the concordance correlation coefficient computed for these data reflects the degree of closeness of the data to this agreement line. In Figure (b), the geometric mean of the Multiplex and ELISA are plotted on the *X*-axis and the ratio between the Multiplex and ELISA data are plotted on the *Y*-axis, after multiplying a correction factor of 2.36 to the ELISA data. The solid line denotes the reference agreement line (ratio = 1) and the dash lines denote the 95% empirical limits of agreement for the ratio of any individual sample.

immunoassay technologies in that the reagents specifically the antibodies are critically important. The antibody screening process and calibrator matrix selection were not described, but considerable time was devoted to these activities. Several lots of pooled human sera were evaluated for matrix interference. The production lot that demonstrated the best performance characteristics was selected and a large quantity was purchased to minimize the effects of inter-lot differences. One consideration that was required for selection of the antibodies was recognition of the World Health Organization reference standard. Mire-Sluis documented a lack of agreement in cytokine results obtained in different laboratories from the same samples. One possible explanation for the lack of agreement was the absence of a universal standard [17]. Therefore, each antibody in the kit recognized the WHO reference standard. Antibodies were also selected

for a lack of a high dose hook effect, and analytical specificity as described in Section 3.

After optimizing the procedure with respect to specificity, accuracy, precision, and linearity, the method was transferred to Linco Research Laboratories. Linco scaled the procedure up and prepared kits to pre-defined specifications. The production of the ready to use kits was essential for large-scale clinical analysis. The kit design minimized the need for manual reagent preparation, which facilitated the adaptation to a diluting workstation.

The analytical sensitivity, precision, accuracy, and linearity were characterized in a pre-study validation. The purpose of the pre-study validation was to predict the performance in-study of quality control samples. Indeed, the pre-study validation precision estimates were generally predictive of the in-study values. For example the TNFα pre-study precision estimate was 9.3–12.0%CV and the in-study was 11.3–13.0%CV. IL-6 was the least predictive; the pre-study values were 5.7–11.0%CV and the in-study values were 11.3–17.7%CV. The accuracy of such estimates could be improved by increasing the number of batches and decreasing the number of replicates per batch to more accurately reflect the variability of inter-assay analysis. Nevertheless, the pre-study validation process was effective for defining the observed in-study performance. Freeze–thaw stability is another important factor to assess in pre-study validation for multiplex analysis. Samples are likely to be assayed multiple times do to failed runs or because the values are outside the limits of quantitation. In this study we defined criteria a priori for acceptable stability that included variability and the % change from control. In the case of IL-8 it is obvious that the criteria were too stringent and IL-8 was stable at four freeze–thaw cycles. The decision was made to adhere to the pre-set criteria and only assay IL-8 samples a total of three times. A thorough assessment of freeze–thaw stability was conducted by Thavasu et al. [18] for three of the five analytes tested in this assay. Their results indicated that the sera of these analytes were stable up to six freeze–thaws for IL-1β, TNFα and IL-6. Thavasu's results are in contrast to these results that indicated IL-1β lost stability after two freeze–thaw cycles and TNFα after three freeze–thaw cycles. One likely explanation for the discrepancy is that Thavasu utilized different antibody pairs. Therefore these results are very specific for the antibody pairs tested and should always accompany validation of new reagents. There is no well-established reference material for defining the analytical accuracy of cytokine assays, and there is no gold standard method for method comparison; however, ELISA has become the most applied method for cytokine quantification. Therefore a comparison of IL-6 ELISA with the Luminex assay yielded a 2.36-fold difference between the two measurements. This systematic (constant) bias is typical when comparing assay formats that do not use the same reference standards, and when the reference standard matrix is not identical to the test sample matrix. Nevertheless, a concordance correlation of 84.5% is impressive given that different laboratories made the measurements with different technology platforms, different antibody pairs, and different lots of standard. After adjusting for the systematic bias/shift (by multiplying the ELISA results by 2.36), the concordance correlation between the ELISA and Luminex results becomes 94.5%. This level of correlation between the multiplex immunoassay and conventional single analyte ELISA supports the concept that cytokine profiling through multiplex testing is practical analytically.

Simultaneous quantification of multiple cytokines over time provides useful mechanistic information in a cost-effective manner. Despite these advantages, multiplex methods introduce a range of development and operational concerns. Examples of these concerns include complexities in the preparation of analytical worklists, data reviews, data management and data approvals. In essence, previous bottlenecks in the sample analyses shifted most notably to data-handling activities. While these concerns can be addressed, the lack of solutions for these operational concerns precluded optimal time and cost benefits from the application of multiplex technology. At present, we are developing approaches to automate and simplify repetitive tasks.

Despite the limitations above, multiplexing technology offers four very important advantages that will inevitably make it a common assay platform in the future. The first is the limited sample volume required to obtain multiple results. In animal studies, pediatric, and critically ill patients, sample volume requirements often preclude the analysis of less well-characterized analytes. Accordingly, this technology opens the possibility to measuring a far broader spectrum of novel biomarkers. Another advantage is the decreased demand for labor and supplies. Since, the assay measures multiple analytes simultaneously, the consumables and labor time are reduced by a factor of the number of analytes. The third advantage is reduced cost of reagents. Currently a 10 analyte multiplex kit costs approximately US$ 1300/96 wells or US$ 130/analyte/96 wells. This is a much more cost-effective option than the single analyte ELISA that values at US$ 500/96 wells. The real cost savings will be obtained when the pre-analytical and post analytical bottlenecks are eliminated. The final advantage is the scalability the technology. Because the system is open and 100 bead classifications exist, as reagents for new biomarkers become available a significantly expanded library of biomarkers will be available. Assuming that no limiting cross-reactivity occurs, one could envision panels to broadly address clinical and research applications.

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