Published in final edited form as:
*Cytokine*. 2009 January ; 45(1): 44–49. doi:10.1016/j.cyto.2008.10.014.

# Reproducibility of serum cytokines and growth factors

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## Abstract

**Background—**In most studies, circulating biomarkers are usually assessed from a single sample, assuming that this single measurement represents the long-term biomarker status of the individual. Such an assumption is rarely tested although it may not be valid for all biomarkers. The objective of this study was to investigate the temporal reproducibility of a panel of cytokines and growth factors.

**Methods—**Thirty-five postmenopausal women with two annual visits and 30 premenopausal women with three annual visits were randomly selected from the participants in an existing prospective cohort. A total of 23 serum cytokines, nine growth factors and C-reactive protein (CRP) were measured using the Luminex xMap™ technology. In addition, for eight biomarkers, regular and high sensitivity (hs) assays were compared.

**Results—**The biomarkers with adequate (>60%) detection rates and acceptable (≥0.55) intra-class correlation coefficients (ICCs) were: hsIL-1β, IL-1RA, hsIL-2, hsIL-4, hsIL-5, hsIL-6, hsIL-10, IL-12p40, hsIL-12p70, hsTNF-α, TNF-R1, TNF-R2, CRP, HGF, NGF, and EGFR. The remaining biomarkers either had low temporal reproducibility or were undetectable in more than 40% of samples.

**Conclusions—**The results suggest that 16 of the 41 biomarkers measured with Luminex technology showed sufficient sensitivity and temporal reproducibility in sera.

### Keywords

Cytokines; C-reactive protein; Growth factors; Interleukins; Reproducibility



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## 1. Introduction

Cytokines and growth factors are critical regulators of immune responses, inflammatory reactions, and the processes of angiogenesis, apoptosis and cell proliferation that play important roles in chronic diseases such as coronary heart disease, diabetes, and cancer [1–5].

When blood samples are collected after the onset of disease, as in retrospective case-control studies, measurements of cytokines are meaningless as predictors of disease risk because of the potential for "reverse causation," i.e., the disease process may have altered prior cytokine levels. For both economic and practical reasons, in most prospective cohort studies only one blood sample is collected per study subject, and exposure-disease associations are usually based on a single measurement. In order for a measurement to be useful, it should reflect the long-term cytokine level for the individual and not mere short-term variability, so the temporal reproducibility of measurements is important to establish before conducting a study of cytokine-disease associations. The degree to which a single measurement reflects the long-term average biomarker level for an individual, relative to others, depends on the between-person variability of these levels over time relative to the within-person variability, as indexed by the intraclass correlation coefficient (ICC) [6].

Several previous studies have assessed the temporal reproducibility for selected cytokines and growth factors in serum [7–15], but these studies have had various limitations: small sample size, assessment of only short-term variability, assessment of only a small number of biomarkers, or use of poor measures of reproducibility. Recent advances in analytical methods such as the Luminex technology allow simultaneous measurement of multiple biomarkers reducing costs, labor, and sample volume requirements [16], which is particularly important for prospective cohorts with banked biological specimens. Two recent studies have used this method to evaluate reproducibility of adipokines and other obesity-related biomarkers, including some cytokines such as IL-1β, IL-6, IL-8, and TNF-α, as well as growth factors such as NGF and HGF [14,15]. However, data on the reproducibility of other cytokines and growth factors measured using Luminex are practically absent.

In the present study, we assessed the reproducibility of a number of cytokines and growth factors in serum from annually drawn blood specimens in a subset of participants from the NYU Women's Health Study (NYUWHS), a large prospective cohort. We measured 23 serum cytokines (IL-1α, IL-1β, IL-1RA, IL-2, IL-2Rα, IL-4, IL-5, IL-6, IL-6R, IL-7, IL-8, IL-10, IL-12p40, hsIL-12p70, IL-13, IL-15, IL-17, TNF-α, TNF-R1, TNF-R2, IFN-α, IFN-γ, sCD40L), nine growth factors (GMCSF, EGF, bFGF, GCSF, HGF, VEGF, TGF-α, NGF, EGFR), and CRP in samples collected over a 2-year period. The biomarkers for this study were selected on the basis of the functional role in inflammatory processes, as well as the availability of commercial kits. In addition, for eight of the biomarkers (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, TNF-α, IFN-γ), both regular and high-sensitivity assays were performed, resulting in 41 measurements for each sample that were analyzed for the current report.

## 2. Materials and methods

### 2.1. The New York University Women's Health Study (NYUWHS)

Between March 1985 and June 1991, 14,274 women 35–65 years old were enrolled at a mammography screening center in New York City. The cohort was restricted to women who in the preceding 6 months were neither pregnant nor treated with hormones. After signing the informed consent at the time of enrollment, and at annual screening visits thereafter, subjects were asked to complete questionnaires on lifestyle and dietary factors and to provide 30 mL of non-fasting peripheral venous blood, drawn using plastic collection tubes without anticoagulant. After drawing, tubes were kept covered at 20 °C for approximately 20 min and

then at 4 °C for 60 min to allow clot retraction. Samples were then centrifuged at 3500 rpm for 15 min, and serum was partitioned into 1-mL aliquots in capped plastic vials within 2 h after separation. Labeled aliquots were immediately stored at −80 °C for future analyses. Fifty-one percent of the cohort members donated blood on more than one occasion, providing the feasibility of doing a reproducibility study.

### 2.2. Reproducibility study design

Subjects were selected at random among NYUWHS participants who fulfilled the criteria listed below: repeated blood donations (at least 2 yearly samples for postmenopausal women and at least 3 yearly visits for premenopausal women); large number of aliquots left; no diagnosis of any cancer (except non-melanoma skin cancer) or cardiovascular disease; no use of any exogenous sex hormones at the time of any of the selected blood donations. Subjects who had been included as cases or controls in any previous nested case-control study were not eligible. In addition, information on the menopausal status and the phase of menstrual cycle for premenopausal women at the time of each blood donation had to be available for the subjects to be selected. Women were classified as post-menopausal if they reported: (a) the absence of menstrual cycles in the previous 6 months; or (b) a total bilateral oophorectomy; or (c) a hysterectomy without total oophorectomy and their age was 52 years or older. Women were classified as premenopausal if they reported at least one menstrual cycle during the past 6 months prior to enrollment. In addition, for each premenopausal woman, the phase of menstrual cycle was calculated from the date of next menstruation, which was obtained from mail-back calendars distributed at the time of blood drawing. Based on the number of days before next menstrual period, a premenopausal women was considered to be in luteal phase (0–11 days), ovulatory (12–16 days), or follicular (≥17 days) phase of the cycle at the time of blood donation.

The annual serum samples of a given subject were collected at approximately same time of the year to minimize the effect of potential seasonal variation of serum markers. Since serum levels of some cytokines may be influenced by sex hormones [17], groups of postmenopausal and premenopausal women were selected. Among women meeting eligibility criteria, we randomly selected 35 postmenopausal women with two yearly samples and 30 premenopausal women with three yearly samples. With 65 subjects and an average 2.45 samples per subject, the expected width of the 95% confidence interval of the ICC was desirably narrow (less than ± 0.15) for ICC ≥0.55. For quality control, random duplicate samples of five premenopausal and five postmenopausal women were selected and analyzed on the same well-plate as the matching samples. Samples' labels contained only sample numbers to ensure blinding of the laboratory personnel.

### 2.3. Luminex assay specifications and procedure

Serum 1-mL aliquots which had not been previously thawed were packed in dry ice and sent to the laboratory. Cytokines and growth factors were analyzed using xMap™ technology which combines the principle of a sandwich immunoassay with fluorescent-bead-based technology allowing multiplex analysis of up to 100 different analytes in a single microtiter well [18]. Soluble CD40L, TGF-α, NGF, and CRP were measured using kits provided by Linco/Millipore Research (Billerica, MA); IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, TNF-α, and IFN-γ were measured using high-sensitivity (hs) kits from Linco/Millipore Research; EGFR was measured using an in-house assay; and all other cytokines and growth factors were measured using kits from Biosource International (Camarillo, CA). The xMap™ serum assays were done in 96-well microplate format according to the protocols. A filter-bottom, 96-well microplate (Millipore, Billerica, MA) was blocked for 10 min with PBS/bovine serum albumin. To generate a standard curve, 5-fold dilutions of appropriate standards were prepared in serum diluent. Standards and patient sera were pipetted at 50 μL per well and mixed with 50 μL of the bead mixture. The microplate was incubated for 1 h at room temperature on a microtiter

shaker. Wells were then washed twice with washing buffer using a vacuum manifold. Phycoerythrin (PE)-conjugated secondary antibody was added to the appropriate wells and the wells were incubated for 45 min in the dark with constant shaking. Wells were washed twice, assay buffer was added to each well, and samples were analyzed using the Bio-Plex suspension array system (Bio-Rad Laboratories, Hercules, CA). All pipetting procedures were performed by a robotic liquid handler (LabStar, Hamilton). Analysis of data was done using four-parametric-curve fitting [19]. The lower limits of detection (LLD) and the coefficients of variation (CVs) for individual biomarkers are presented in Table 2.

### 2.4. Statistical analysis

All analyses were performed on natural-logarithm-transformed values in order to reduce the positive skewness of the raw data. The temporal reproducibility was estimated by the intraclass correlation coefficient, which is defined as the proportion of the total variability that is due to between-subject variability. The variance components were estimated with a random effects one-way analysis of variance model, using the SAS procedure MIXED. Exact 95% confidence intervals (CIs) for the ICCs were calculated as described by McGraw and Wong [20]. We established a *priori* that cytokine variables worthy of future consideration should be detectable in at least 60% of the samples in order to calculate meaningful ICCs and should have an ICC of at least 0.55 based on our previous experience [21–23] and recommendations in the literature [24].

Mixed-effects models were also fitted to assess the effects of covariates, including order of blood donation (1st, 2nd or 3rd), blood storage time, age at blood donation, menopausal status (pre/post), phase of menstrual cycle for premenopausal women, body mass index (BMI), race/ethnicity, medication use during a four-week period before the blood donation, alcohol consumption at baseline (0, 1–5, 6–10, >10 standard drinks/week), and smoking status at baseline (non-smoker, current smoker, past smoker) on the levels of the biomarkers.

Because levels of a biomarker from annual samples from the same subject were highly correlated, the bootstrap method was used to calculate the Spearman correlation coefficients ($r$) between different biomarkers. In brief, one sample was randomly selected for each subject to create a group of 65 mutually independent samples, the $r$ between the cytokines/growth factors was calculated based on these 65 samples, and then this step was repeated 100 times and the average $r$ was reported. All analyses were performed using SAS 9.1 (SAS Institute, Cary, NC). All $p$ values are two-sided.

## 3. Results

### 3.1. Characteristics of study subjects

The mean age of the 65 subjects was 50.8 years and 85% of them were of European descent (Table 1). The mean age at first blood donation for premenopausal and postmenopausal women was 43.3 and 57.2 years, respectively. The mean body mass index at time of the first blood donation for premenopausal and postmenopausal women was 23.9 and 24.2 $kg/m^2$, respectively. Mean durations in storage of the serum samples from premenopausal women were 20.0, 18.8, and 17.5 years for visits 1, 2, and 3, respectively, and from postmenopausal women were 20.3 and 19.3 years for visits 1 and 2, respectively.

### 3.2. ICCs and their 95% CIs for the biomarkers

Table 2 presents descriptive statistics and ICCs for the 20 serum cytokines and growth factors which were detectable in more than 60% of the serum samples analyzed. Sixteen of these 20 biomarkers (hsIL-1β, IL-1RA, hsIL-2, hsIL-4, hsIL-5, hsIL-6, hsIL-10, IL-12p40, hsIL-12p70, hsTNF-α, TNF-R1, TNF-R2, CRP, HGF, NGF, and EGFR) had ICCs greater than 0.55,

indicating they are reasonably reliable over time. For 21 other biomarkers (IL-1α, IL-1β, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-10, IL-13, IL-15, IL-17, TNF-α, IFN-α, IFN-λ, hsIFN-λ, TGF-α, GMCSF, GCSF, bFGF, and VEGF), more than 40% of all the samples were below the lower limit of detection (LLD). A biomarker which is below the detection limit in more than 40% of the subjects may still be used in epidemiologic studies. However, it is unclear how meaningful is the ICC for such a biomarker, therefore ICCs for these 21 biomarkers are not presented. Detection rates were very similar from visit to visit for most of the assays and the maximal difference in detection rates from visit to visit was observed for regular IL-6 (22%) from visit 1 to visit 3.

### 3.3. Effects of covariates on the levels of biomarkers and their ICCs

Most of the biomarkers showed no associations with the covariates we considered: age at blood donation, order of blood donation, blood storage time, menopausal status, phase of menstrual cycle (for premenopausal women), BMI, race/ethnicity, medication use, alcohol consumption at baseline and smoking status at baseline. Of particular interest, smoking and phase of menstrual cycle were not significantly correlated with any of the examined markers and had no impact on the ICCs. Limiting the analyses to subjects of European descent did not substantially change any ICC. Consistent with other studies, positive correlations with BMI were found for CRP ($r = 0.51$) and IL-6 ($r = 0.26$) (but not for hsIL-6, $r = 0.10$). Adjusting for BMI, however, had little impact on the ICCs: the unadjusted ICC of 0.73 for CRP became 0.65 (95% CI: 0.51–0.76), and 0.48 for IL-6 became 0.45 (0.25–0.62). Use of NSAIDs and other medications was associated with significantly lower levels of hsIL1β ($p < 0.02$). Alcohol drinking at baseline was negatively associated with levels of hsTNF-α ($p = 0.02$). Age at sample collection was positively associated with hsTNF-α ($p < 0.0001$), and with TNFR2 levels ($p < 0.0001$). Postmenopausal status, after adjustment of age at sample collection, was positively associated with hsTNF-α and with TNFR2 levels ($p < 0.01$ and $p = 0.02$, respectively). Post-menopausal status was also positively associated with IL-5 levels ($p = 0.04$). No significant associations were observed in cytokine levels by phase of cycle in premenopausal women. Controlling for these covariates did not substantially change the respective ICCs (data not shown).

### 3.4. Correlations between biomarkers

The bootstrap Spearman correlation coefficients between bio-markers are listed in Table 3. The highest correlations (0.8–0.9) were observed between hsIL-4, hsIL-5, hsIL-6 and NGF. High-sensitivity IL-1β was correlated with IL-1RA, hsIL-2, and hsIL-12p70; IL-1RA was correlated with hsIL12p40 and TNF-R1; hsIL-2 with hsIL-12p70; and hsIL-10 with hsTNF-α and hsIL-12p70. As expected, CRP was positively correlated with hsIL-6 and TNF-α. The positive correlation between CRP and hsIL-4 was, however, unexpected and could be explained by high correlation observed between IL-4 and IL-6 ($r = 0.87$) in this study.

### 3.5. Comparison between regular and high-sensitivity assays

We measured IL-1b, IL-2, IL-4, IL-5, IL-6, IL-10, TNF-α and IFN-λ using both high-sensitivity and regular assays. Regular assays did not have the ability to detect most of the samples for all these cytokines, whereas high-sensitivity assays substantially improved the detectability (Table 4). Correlations between the measured values of regular and high-sensitivity assays were low, with the highest being 0.39 for IL-2.

## 4. Discussion

We used Luminex xMap™ technology to investigate the ability of a single measurement of several cytokines or growth factors to represent a subject's long-term average levels, relative to other subjects. To our knowledge, this is the first study to measure the temporal

reproducibility of a large number of cytokines and growth factors using Luminex methodology. The results demonstrated that 16 out of the 41 biomarkers measured were quite stable in a woman over time, so that a single measurement of these biomarkers represents the average level reasonably well over 2-year period. Therefore, these biomarkers, measured using the Luminex xMap™ method, are good candidates for epidemiologic studies with prospectively collected serum samples.

The selection of 0.55 as the acceptable lower limit for temporal reproducibility is based on our own previous experience with sex steroid hormones reproducibility [21–23] and recommendations in the literature [24]. Intraclass correlation coefficients on the order of 0.5–0.7 appear to be typical for the studies of postmenopausal circulating hormones [22,25], which nevertheless were able to show significant associations with breast cancer risk [26]. In choosing an acceptable limit for temporal reproducibility, investigators should consider the availability of other biomarkers and/or laboratory methods as well as the expected attenuation in relative risks. For example, assuming that the true relative risk for a chronic disease associated with a biomarker is 2.0, an ICC of 0.80 would lead to an observed relative risk of 1.74 [$RR_{observed} = \exp(\ln RR_{true} \times ICC)$], whereas ICCs of 0.55 and 0.40 would lead to observed relative risks of 1.46 and 1.32, respectively [27]. Therefore, low ICCs lead to observed relative risks closer to unity, which therefore could mask true exposure-disease associations.

For 21 biomarkers, more than 40% of the 160 samples were below the lower limit of detection. The possible reasons for low detection rates of several biomarkers could be low levels of these markers in sera from “healthy” women, low sensitivity of the regular assays, or low stability and potential degradation of certain proteins during long-term storage at −80 °C.

The circulating levels of cytokines, CRP and growth factors are thought to be affected by a wide range of covariates, including age, BMI, medication use, race/ethnicity, smoking, alcohol use, hormone replacement therapy, phase of menstrual cycle and menopausal status [9,17, 28–31]. Although we found correlations of these covariates with several cytokines, growth factors or CRP, in no case did adjustment for the covariates alter the ICCs substantially. We could not assess the effect of exogenous hormones on the biomarkers, because women who had taken hormones in the 6 months prior to blood donation were excluded from our study. Of note, age at blood donation was not associated with most of the biomarkers, perhaps because of the narrow age range of the study subjects, and phase of the menstrual cycle had no apparent impact on biomarker levels.

High-sensitivity ELISA or Luminex xMap method have been used in previous studies to measure cytokines such as IL-6 and TNF-α [9,13] or CRP [15]. However, this is the first study, to our knowledge, to directly compare high-sensitivity assays with regular assays, for the same analytes using the same xMap method. As expected, compared to regular assays, the high-sensitivity assays led to higher proportions of samples above the detection limit. The correlations between biomarkers measured by regular and high-sensitivity Luminex xMap assays were generally low in our study. This is not uncommon, as low correlation have been reported for the concentrations of an analyte in the same specimen measured using different commercial assays [32]. Significant variations in biomarker concentrations could be due to differences in the capture and reporter antibodies used [33,34], which can result in differential detection of various subforms of a particular bio-marker or to differences in cross-reactivity with other serum proteins [35]. Overall, using high-sensitivity assays greatly improved the detection rates and reproducibility of markers using the Luminex method.

We also explored the correlations among measured biomarkers. The highest correlation coefficients were observed among NGF, IL-4, IL-5 and IL-6. NGF, the best characterized member of the neurotrophins, has the effects on both the peripheral nervous system and non-

neuronal cells, including lymphocytes, granulocytes and monocytes [36]. NGF levels have been reported to be increased in humans with allergic diseases [37] and it has been suggested to be viewed as a Th2 cytokine [38]. Consistent with this, we observed very high correlations between NGF and some Th2 cytokines including IL-4, IL-5, and IL-6, as well as a moderate high correlation with IL-10, which are all important immune mediators in allergic conditions. Similarly, the levels of IL-12p70 (bioactive form of IL-12), the key player in induction of Th1 immune responses, were highly correlated with levels of other Th1 cytokines, such as IL-2. The high correlation between IL-1RA and TNF-α observed in this study can be supported by the involvement of TNF-α in the production of IL-1RA [39]. IL-6, TNF-α and IL-1β are known to be the primary regulators of the hepatic synthesis of acute phase proteins such as CRP [40]. Our data show that the serum IL-6 and TNF-α were positively, albeit moderately, correlated with CRP ($r$ = 0.25 and 0.27, respectively). However, contrary to expectation, but consistent with a recent report [15], the IL-1β was negatively correlated with CRP. These observations require replication in future studies.

Our study was relatively large compared to most of the previous reproducibility studies of cytokines and growth factors. We used serum samples that were never previously thawed and the laboratory personnel were blinded as to the identity of the samples to avoid bias. Our study also had some limitations. Among limitations of the study was that data on autoimmune and infectious conditions were not collected at baseline. However, the NYUWHS cohort participants were apparently healthy middle-age women at blood donation, and the possibility of serious viral or bacterial infections in this population is expected to be low. The study assessed relatively short-term reproducibility using samples collected 2–3 years apart. Studies of long-term reproducibility would be of great interest. Also, our study included only women 35 or older, limiting the generalizability of the results mainly to postmenopausal and perimenopausal women.

In conclusion, we found that serum concentrations of 16 biomarkers (hsIL-1β, IL-1RA, hsIL-2, hsIL-4, hsIL-5, hsIL-6, hsIL-10, IL12p40, hsIL-12p70, hsTNF-α, TNF-R1, TNF-R2, CRP, HGF, NGF, and EGFR), measured using the Luminex method, are stable in women over a 2-year period. This suggests that a single measurement of these biomarkers may be used for risk assessment in studies using frozen samples collected prospectively.

## Acknowledgments

The authors thank Lynne Quinones and Yelena Afanasyeva for technical assistance. This research was supported by the National Institutes of Health Grants R01 CA98661 and R03 CA96428, and by Cancer Center Grant CA16087 from the National Cancer Institute and the NIEHS Center Grant ES00260.

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**Table 1**

Characteristics of study subjects.

| | Pre-menopausal women | Post-menopausal women | All subjects |
|---|---|---|---|
| Number of subjects | 35 | 30 | 65 |
| Age at first blood donation[a], years | 43.3 (±3.6) | 57.2 (±4.9) | 50.8 (±8.2) |
| *Storage time,[a] years* | | | |
| 1st sample | 20.6 (±1.2) | 20.9 (±1.1) | 20.4 (±1.2) |
| 2nd sample | 19.3 (±1.3) | 19.9 (±1.1) | 19.6 (±1.2) |
| 3rd sample | 18.1 (±1.3) | - | 18.1 (±1.3) |
| BMI at time of the first blood donation,[a,b] kg/m$^2$ | 24.2 (±4.6) | 24.6 (±3.1) | 24.4 (±3.9) |
| *Race/ethnicity,[b] n* (%) | | | |
| African-American | 2 (8.7%) | 2 (6.7%) | 4 (7.8%) |
| European descent | 19 (82.6%) | 26 (86.7%) | 45 (84.5%) |
| Latina and others | 2 (8.6%) | 2 (6.6%) | 4 (7.8%) |
| *Smoking status at baseline,[b] n* (%) | | | |
| Non-smoker | 12 (44.4%) | 14 (43.8%) | 26 (44.1%) |
| Current smoker | 6 (22.2%) | 5 (15.6%) | 11 (18.6%) |
| Past smoker | 9 (33.3%) | 13 (40.6%) | 22 (37.3%) |
| *Alcohol consumption at baseline (standard drinks/week),[b] n* (%) | | | |
| None | 11 (45.8%) | 20 (64.5%) | 31 (56.4%) |
| 1–5 | 6 (25.0%) | 8 (25.8%) | 14 (25.4%) |
| 6–10 | 5 (20.8%) | 2 (6.5%) | 7 (12.7%) |
| >10 | 2 (8.4%) | 1 (3.2%) | 3 (5.5%) |

[a] Mean ± standard deviation.

[b] Numbers of subjects with missing values for the covariates were as follows: BMI (1), race/ethnicity (12), smoking status at baseline (6), and alcohol consumption at baseline (10).

**Table 2**

Lower limit of detection (LLD), intra-batch coefficients of variation, percentage of samples above detection limit, intraclass correlations (95% CIs), and medians (25th and 75th percentiles) of serum biomarkers measured by the Luminex xMap™ method.[a]

| Biomarker | LLD | Intra-batch CV (%) | % of samples above LLD | ICC (95%CI) | Median (25th–75th percentile) |
|---|---|---|---|---|---|
| hsIL-1β (pg/mL)[b] | 0.06 | 8.6 | 89 | 0.86 (0.78–0.91) | 1.9 (0.7–5.7) |
| IL-1RA (ng/mL)[c] | 0.03 | 3.7 | 100 | 0.57 (0.41–0.70) | 0.4 (0.3–0.7) |
| hsIL-2 (pg/mL)[b] | 0.16 | 19.6 | 80 | 0.81 (0.71–0.88) | 1.4 (0.3–5.8) |
| hsIL-4 (pg/mL)[b] | 0.13 | 9.1 | 89 | 0.92 (0.87–0.95) | 14.4 (4.0–84.5) |
| hsIL-5 (pg/mL)[b] | 0.01 | 6.4 | 72 | 0.89 (0.81–0.93) | 0.1 (<LLD-0.9) |
| hsIL-6 (pg/mL)[b] | 0.10 | 3.8 | 100 | 0.92 (0.88–0.95) | 7.6 (2.8–27.2) |
| hsIL-10 (pg/mL)[b] | 0.15 | 5.1 | 90 | 0.75 (0.63–0.84) | 3.9 (2.0–7.5) |
| IL-12p40 (ng/mL)[c] | 0.015 | 1.8 | 100 | 0.60 (0.45–0.73) | 0.2 (0.2–0.3) |
| hsIL-12P70 (pg/mL)[b] | 0.11 | 4.9 | 62 | 0.83 (0.74–0.89) | 0.4 (<LLD-3.5) |
| hsTNF-α (pg/mL)[b] | 0.05 | 2.2 | 100 | 0.88 (0.82–0.92) | 4.3 (3.2–5.3) |
| TNF-R1 (ng/mL)[c] | 0.015 | 1.7 | 100 | 0.68 (0.55–0.78) | 1.7 (1.3–2.3) |
| TNF-R2 (ng/mL)[c] | 0.015 | 0.4 | 100 | 0.80 (0.71–0.87) | 0.7 (0.6–0.9) |
| CRP (mg/L)[b] | $6\times10^{-6}$ | 2 | 99 | 0.73 (0.62–0.82) | 2.2 (1.0–5.6) |
| HGF (ng/mL)[c] | 0.01 | 3.6 | 99 | 0.60 (0.45–0.72) | 0.2 (0.1–0.3) |
| NGF (pg/mL)[b] | 2.5 | 9 | 93 | 0.95 (0.93–0.97) | 19.8 (7.7–86.5) |
| EGFR (ng/mL)[d] | 0.0188 | 0.4 | 99 | 0.67 (0.54–0.78) | 34.6 (30.8–38.2) |
| sIL-6R (ng/mL)[c] | 0.0024 | 0.4 | 86 | 0.52 (0.36–0.67) | 71.4 (39.4–124.0) |
| IL-8 (pg/mL)[c] | 1.3 | 1.3 | 60 | 0.02 (0–0.21) | 4.2 (<LLD-14.2) |
| sCD40L (ng/mL)[b] | 0.00113 | 1.9 | 99 | 0.44 (0.26–0.59) | 5.2 (3.9–6.5) |
| EGF (pg/mL)[c] | 15 | 2.4 | 60 | 0.21 (−0.03–0.44) | 27.9 (<LLD-84.1) |

*Note:* <LLD, Less than the lower limit of detection.

[a]Limited to the biomarkers for which more than 60% of all the samples were detectable. Twenty-one markers for which more than 40% of all the samples were undetectable (IL-1α, IL-1β, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-10, IL-13, IL-15, IL-17, TNF-α, IFN-α, IFN-γ, hsIFN-γ, TGF-α, GMCSF, GCSF, bFGF, and VEGF) are not included.

[b]Kits from Linco/Millipore.

[c] Kits from Biosource.

[d] Kit developed in-house.

**Table 3**

Spearman correlation coefficients between cytokines.[a,b]

| | hsIL-1β | IL-1RA | hsIL-2 | hsIL-4 | hsIL-5 | hsIL-6 | hsIL-10 | IL-12p40 | hsIL-12p70 | hsTNF-α | TNF-R1 | TNF-R2 | CRP | HGF | NGF |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| hsIL-1β | 1.00 | | | | | | | | | | | | | | |
| IL-1RA | **0.40** | 1.00 | | | | | | | | | | | | | |
| hsIL-2 | **0.56** | **0.32** | 1.00 | | | | | | | | | | | | |
| hsIL-4 | **0.12** | −0.03 | **0.30** | 1.00 | | | | | | | | | | | |
| hsIL-5 | **0.23** | 0.00 | **0.33** | **0.84** | 1.00 | | | | | | | | | | |
| hsIL-6 | **0.21** | 0.02 | **0.28** | **0.87** | **0.79** | 1.00 | | | | | | | | | |
| hsIL-10 | **0.21** | −0.06 | **0.31** | **0.28** | **0.29** | **0.37** | 1.00 | | | | | | | | |
| IL12p40 | **0.14** | **0.56** | 0.15 | − **0.18** | −0.08 | −0.03 | 0.14 | 1.00 | | | | | | | |
| hsIL12p70 | **0.51** | **0.23** | **0.49** | **0.19** | **0.35** | **0.32** | **0.60** | 0.11 | 1.00 | | | | | | |
| hsTNF-α | 0.06 | −0.04 | **0.24** | **0.31** | **0.27** | **0.36** | **0.49** | 0.11 | **0.33** | 1.00 | | | | | |
| TNF-R1 | **0.25** | **0.72** | **0.31** | **0.19** | 0.17 | **0.19** | 0.04 | **0.40** | **0.15** | 0.05 | 1.00 | | | | |
| TNF-R2 | −0.02 | 0.02 | 0.09 | 0.17 | 0.03 | **0.22** | **0.17** | **0.16** | 0.10 | **0.38** | **0.23** | 1.00 | | | |
| CRP | − **0.17** | −0.08 | 0.07 | **0.28** | 0.09 | **0.27** | 0.16 | −0.01 | 0.06 | **0.25** | 0.02 | **0.12** | 1.00 | | |
| HGF | −0.03 | **0.45** | 0.13 | 0.02 | −0.01 | **0.07** | 0.00 | **0.30** | **0.14** | **0.21** | **0.32** | 0.12 | **0.24** | 1.00 | |
| NGF | **0.09** | −0.02 | **0.23** | **0.85** | **0.75** | **0.82** | **0.18** | −0.03 | 0.09 | **0.27** | **0.17** | **0.14** | **0.24** | −0.01 | 1.00 |
| EGFR | −0.11 | −0.06 | −0.10 | − **0.19** | − **0.20** | − **0.17** | − **0.16** | 0.03 | −0.11 | − **0.19** | −0.05 | **0.18** | −0.02 | 0.07 | − **0.25** |

*Notes:* Mean *r* was calculated using a bootstrap method, in which one sample was randomly selected for each of the 65 subjects, and a correlation coefficient between cytokines was calculated based on these 65 observations. This procedure was repeated 100 times, and an average *r* was calculated and reported here.

[a] The 16 biomarkers from Table 2 with ICC ≥ 0.55 were included in this table.

[b] Statistically significant ($p < 0.05$) Spearman correlation coefficients are marked in bold.

**Table 4**

Detection limits and Spearman correlations between the regular and high-sensitivity assays.

| | Lowest detection level (pg/mL)* | | % of samples above detection limit | | Spearman correlation coefficient |
|---|---|---|---|---|---|
| | Regular assay | High-sensitivity assay | Regular assay (%) | High-sensitivity assay (%) | |
| IL-1β | 15 | 0.06 | 16 | 89 | 0.31 |
| IL-2 | 6 | 0.16 | 39 | 80 | 0.39† |
| IL-4 | 5 | 0.13 | 55 | 89 | 0.03 |
| IL-5 | 3 | 0.01 | 3 | 72 | 0.13 |
| IL-6 | 3 | 0.1 | 38 | 100 | 0.24† |
| IL-10 | 5 | 0.15 | 6 | 90 | 0.20 |
| TNF-α | 10 | 0.05 | 30 | 100 | 0.03 |
| IFN-γ | 15 | 0.29 | 8 | 54 | 0.29 |

*Regular assay kit was from Biosource, and high-sensitivity assay kit from Linco/ Millipore.

†$p < 0.05$.