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Effect of anticoagulants on multiplexed measurement of cytokine/chemokines in healthy subjects

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Abstract

Introduction—Cytokines are humoral regulatory molecules that act together in immunologic pathways. Monitoring cytokines and their variations within physiologic ranges is critical for biomarker discovery. Therefore, we evaluated the performance characteristics of 72 analytes measured by multiplex cytokine immunoassay, with an emphasis on the differences of analytes measured in serum compared to plasma, and, in plasma, on the impact of anticoagulants on the cytokine measurement.

Methods—We used fluorescent bead-based (Luminex) immunoassay kits to simultaneously measure 72 analytes. We tested serum and plasma samples from 11 matched donors. Plasma samples were anti-coagulated with sodium heparin, sodium citrate dextrose and ethylene diamine tetra-acetic acid (EDTA), respectively.

Results—Of the 72 cytokines, 12 were undetectable in all types of specimen samples. Nineteen analytes, including PDGF-bb, IL-4, IL-8, IL-9, FGF-b, PAI-1, CXCL-5, CCL-5, CD40L, EGF, VEGF, IL-2ra, IL-3, SDF-1a, PCT, MCP-3, GIP, IL-16 and fibrinogen, showed significant differences between measurements in serum and all types of plasma, regardless of anticoagulant. Among plasma samples, 10 analytes (eotaxin, SCGF-b, MCP-1, SCF, MIP-1b, VEGF, RANTES, PDGF-b, PAI-1 and ITAC) showed significantly higher concentrations in heparinized plasma compared to citrated and EDTA plasma. IP-10, and CTAK were the only 2 cytokines that presented different concentrations in citrate and EDTA plasma.

Conclusions—With their small volume, low cost per test, and multiplex capacity, Luminex-based cytokine assays have enormous potential utility for screening in epidemiologic studies. In our study, we showed that many cytokines' concentrations differed between serum and plasma samples, and that different anticoagulants used in preparation of plasma samples also affected the measurement of some cytokines. There was no optimal sample preparation that was clearly superior for the measurement of all analytes measured. Ultimately, the utility of cytokine

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Conflict of interest

All authors have no conflict of interest.

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measurement, as biomarker or to monitor the immune system, will depend on attention to detail in the collection and processing of samples in addition to assay precision.

Keywords

cytokines/chemokines; plasma; serum; anticoagulants

1. Introduction

Cytokines and chemokines are small molecules with large roles in modulating immune reactions [1]. The measurement of cytokines permits assessment of the immune response in a disease or in response to a specific therapy. A particular cytokine may exert effects on multiple different types of cells, and multiple cytokines can exert various effects on a single cell type. Historically, most measurements of cytokines and chemokines have been made using single-plex enzyme-linked immunosorbent assay (ELISA) methods. This approach to cytokine measurement, while quite valuable, limited the scope of analytes that could be examined in any given trial, and thus studies of the cytokine network interactions were quite limited, often precluding comprehensive assessment of cytokines and chemokines for such studies.

Multiplex immunoassay technologies to monitor immune responses by measuring biomarkers such as cytokines have become increasingly available; they have the capability to measure up to 100 simultaneously in a minimal amount of biological sample [2]. Determining the accuracy and reliability of the detection method used for each analyte in multiplex assays is key for the use of these assays in clinical trials. While analytical variables, such as matrix effects induced by multiplexing analyte assays, have been widely studied [3, 4], less attention has been paid to pre-analytical variables that can also affect multiplex cytokine assays. These can include assorted elements of the “preparasome”, such as how the specimen is stored, diurnal variations, and type of specimen (such as serum or plasma). Among pre-analytical variables, little is known regarding how the measurement of most cytokines is affected by the anticoagulant used for collection of blood from healthy individuals. In the few studies that have begun to address this issue [5, 6], only a small number of analytes were examined. As a result there are little, if any, data for most cytokines regarding how measurements are affected by anticoagulants. In the present study, we analyzed seventy-two analytes in serum and plasma from healthy individuals. Plasma collection used several anticoagulants, including sodium heparin, acid citrate dextrose, and EDTA. Our primary objective was to evaluate anticoagulant impact on the measured levels of cytokines in the peripheral circulation and to determine the best sample preparation for most cytokine measurements.

2. Materials and Methods

2.1 Samples

Samples were obtained concurrently from 11 healthy donors enrolled in protocol 09-H-0239, approved by the NHLBI institutional review board, and written informed consent was obtained from all participants. To assess cytokine levels in different preparations, we used matched samples collected the same day from the same patient. Table 1 summarizes the specifics of each type of sample collection tubes. Serum samples were isolated 2 hours after collection of blood using serum separating tubes (SST) (Beckton Dickinson, San Jose, CA, USA). Plasma was collected in 3 different ways depending on the type of anticoagulant present in the tube; i) Sodium heparin (“green top tube”; 15 IU/mL $\pm$ 2.5 IU/mL), ii) ethylene diamine tetra-acetic acid (EDTA) (“lavender top tube”; 1.5 mg/mL $\pm$ 0.35 mg/

mL), iii) sodium citrate dextrose (“yellow top tube”; 0.109 mg/mL). Specimens used for this study were subjected to no more than 2 freeze/thaw cycles.

2.2 Laboratory Procedure

We used 6 different kits from 2 manufacturers; 5 kits were from Bio-rad (Hercules, CA, USA) and 1 kit from R&D (Minneapolis, MN, USA). All assays were performed according to Bio-Rad and R&D kit procedures.

Median fluorescence intensities were collected on a Luminex-100 instrument (Luminex, Bio-rad), using Bio-Plex Manager software version 6. Standard curves for each cytokine were generated using the premixed lyophilized standards provided in the kits. Cytokine concentrations in samples were determined from the appropriate standard curve using a 5 Point-regression to transform mean fluorescence intensities into concentrations. Each sample was run in duplicate and the average of the duplicate was used as the measured concentration. Three cytokines were measured in the kits from different vendors, IL-8, eotaxin and IP-10, and their results were presented separately.

2.3 Statistical Analyses

Data obtained with serum/plasma from one donor were considered as one experiment (n). Results are shown as the mean of the observed concentration for each person, the standard of the mean and the percentage of coefficient of variation of the mean. Data were compared by nonparametric analyses with Wilcoxon’s matched pairs test, in which the median was used to calculate significant difference, p values significance level was set as $p=0.05$ and the actual p values were indicated for each series of experiments. Plasma_{heparin} was used as a control, as it is the most common type of anti-coagulant used for plasma preparation.

Statistical calculations were performed using Bio-plex data pro software (version 1.0 (Bio-rad)) and when serum was used as control instead of plasma_{heparin}, p values are showed in the figures. Heat maps were generated after normalization of the data, using $\log_2$ transformation of the observed concentration. The normalized data were then expressed as percentages of the highest value and thus, for each cytokine, with color gradient from low to high concentrations (green to red).

3. Results

3.1 General observations

Numerous differences were observed among the cytokine measurements obtained with serum and the plasma samples collected with different anticoagulants. Table 2 displays a summary of the results obtained for each kit, independent from one another. There was no single method of specimen preparation that was clearly superior for the measurement of all 72 analytes tested. Of these, the following twelve were below the limit of detection in all four types of sample; IL-1a, IL-1b, IL-2, IL-5, IL-10, IL-13, IL-15, GM-CSF, LIF, M-CSF, TNF-a and TNF-b. The failure to detect these analytes in samples obtained from healthy volunteers was consistent with data previously reported by others [2, 7]. Further analysis on the influence of anticoagulants on the measurement of these analytes was therefore not possible.

3.2 Comparison of cytokine measurements in serum specimens to plasma specimens

Comparisons between serum and plasma were conducted by: 1) comparing serum to all plasma types, regardless of anticoagulant, and 2) comparing serum to each type of anti-coagulated plasma.

Comparison of cytokine measurements in serum to all plasma types, regardless of anticoagulant—Thirty-two analytes (CTACK, GLP-1, SAA, CRP, Leptin, C-peptide, SAP, Haptoglobin, Ferritin, Insulin, TRAIL, Resistin, A2M, IFN- α 2, MIF, Glucagon, IL-6, IFN- γ , IL-7, Ghrelin, G-CSF, MIG, Visfatin, GRO α , IL-1 α , MIP-1 α , MIP-1 β , IL-12 (p70), IL-18, tPA, HGF, IL-17) displayed no statistically significant differences when measured in serum or any type of plasma.

By contrast, 19 analytes, including PDGF-bb, IL-4, IL-8, IL-9, FGF-b, PAI-1, CXCL-5, CCL-5, CD40L, EGF, VEGF, IL-2 α , IL-3, SDF-1 α , PCT, MCP-3, GIP, IL-16 and fibrinogen, showed statistically significant differences between measurements in serum or all types of plasma, regardless of anticoagulant, however the values measured were not equivalent in all types of plasma. The majority yielded higher values in serum than plasma (Fig. 1A), with the exception of IL-2 α , IL-3 and SDF-1 α , which were not detectable in serum (Fig. 1B, Table 2) and PCT, MCP-3, IL-16, GIP and fibrinogen, which yielded lower values in serum than plasma (Fig. 1C, Table 2).

Comparison of cytokine measurements in serum to each type of anti-coagulated plasma—The remaining 9 analytes displayed statistically significant differences when measured in serum compared to at least one type of plasma sample, but not to all types of plasma samples (SCGF-b, RANTES, IL-17, eotaxin, b-NGF, IL-12 (p40), MCP-1, SCF, IP-10) (Table 2, Fig. 2).

3.3 Comparison of cytokine levels in plasma specimens collected in different anticoagulants

Significant differences in the measurement of numerous analytes were observed in plasma samples obtained using different anticoagulants. EDTA and citrate yielded similar, but not identical, measurements of most of the 72 analytes assayed, with disparities found only for IP-10 and CTAK (Fig. 3). Plasma obtained using heparin was found to be significantly different from citrate and EDTA for the measurement of 10 analytes; eotaxin (R&D), SCGF-b, MCP-1, SCF, MIP-1 β , VEGF, RANTES, PDGF-b, PAI-1 and ITAC showed significantly higher concentrations in heparin plasma compared to citrate and EDTA plasma. CTAK, tPA, b-NGF showed significantly lower concentrations in heparin plasma compared to citrate and EDTA plasma.

3.4 Heat map representation of the Bio-rad kits luminex

A heat map summarizing merged data from each type of samples was generated after normalization of the concentration using the log₂ of the concentration (Fig. 4). In green, we confirmed that some cytokines were not detectable in all type of specimens, IL-1 α , IL-1 β , IL-2, IL-5, IL-10, IL-13, IL-15, GM-CSF, LIF, M-CSF, TNF- α and TNF- β , identified with an arrow below the heat map. The heat map also allowed us to rapidly identify which type of sample will provide the higher measurement, as for PAI-1, PDGF-bb, RANTES (marked with a yellow circle).

3.5 Comparison of kits from different manufacturers

An 8-plex kit from R&D was run using serum and 3 types of plasma from 20 donors (different donors from those used with the Bio-Rad kits). These data revealed numerous significant differences as noted in Table 2 and Figure 5. This kit also shared three analytes - IL-8, eotaxin and IP-10 - which were measured in Bio-rad kits. Interestingly, for these analytes, the changes observed in the R&D kits were different from those seen in the Bio-rad kits (Fig. 5). IL-8 detection was consistent in both kits, showing a higher yield in serum compared to all plasma. Eotaxin and IP-10 presented discordant results, in both concentration values and yield in sample type. IP-10 from R&D could not be recovered from

serum, and eotaxin from R&D was better detected in heparin plasma samples. With both Bio-rad kits, we observed a statistically significant difference for IP-10 between citrate and serum, and eotaxin in EDTA plasma showing lower yield compare to the other 3 types of samples.

4. Discussion

We assessed variation in the measurement of circulating analytes in blood samples prepared by spontaneous clotting to serum and with three different methods of anticoagulation to create plasma. Regardless of the type of samples used, the best way to limit problems is to immediately start separating the plasma or serum (post clotting) from the cellular elements. An important goal in optimization of multiplex assay is to reduce the matrix effect by using the appropriate sample diluent, thereby decreasing non-specific antibodies interactions [8]. Apart from the matrix effect, we studied the importance of the anticoagulants used for plasma.

The coagulation cascade necessary to produce clotting, and hence serum, is a complex biochemical process and it is not surprising that measurements of analytes made in plasma are generally not reflective of serum levels and thus are not interchangeable. Several studies have shown that plasma samples and serum collected from subjects at the same time yield different measurements of some cytokines [2, 7, 9]. Serum preparation includes removal of fibrinogen, platelets and other circulating proteins from the plasma that could influence the presence or detection of an analyte on such a sample. Also, during the cascade of coagulation, activation of cellular elements can release inflammatory mediators, that may affect cytokine levels [10, 11].

The reason for the differences in cytokine concentrations observed among the different types of plasma is not known. EDTA and citrate both remove calcium to prevent coagulation while heparin acts instead by binding to antithrombin III[12]. Indeed, we observed only two differences in cytokine measurements between EDTA and citrate, while more differences were noted when comparing heparin to both EDTA and citrate.

Previously published work showed difference in CRP and IL-6 when comparing plasma to serum [9], but we did not observe such higher levels of IL-6 and CRP in plasma. It is difficult to speculate on the reason why we have different results, and the nature of the antibodies used in this assay cannot be ruled out, as the clones of antibodies used for IL-6 and CRP detection in the Bio-rad kits are not provided by the manufacturer.

In agreement with the literature, our results indicate that analytes such as CD40L, which are secreted by platelets upon activation during the coagulation reaction, are found at a higher concentration in serum [13–17]. It is been previously reported that EDTA plasma is not appropriate for measurement of platelet-derived cytokines [18].

Our results emphasize the need to consider experimental design carefully when a study requires measurement of cytokines in the peripheral circulation. The quality of cytokine measurements begin when the sample is collected and the choice of anticoagulant should be carefully considered. Additional factors such as sample preparation and time and temperature of storage have been reported to be important as well (19). Under no circumstances should cytokine measurements in a single study use samples collected in different anticoagulants or a combination of plasma and serum. Ultimately, the utility of cytokine measurement will depend on rigorous attention to detail in the collection and processing of samples in addition to assay precision.

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Highlights

- The measurement of cytokines can be affected collection in either plasma or serum.
- In plasma, the choice of anticoagulant affects the measurement of some cytokines.
- There is not one optimal type of sample for measurement of all cytokines.

Figure 1. Comparison of measured concentrations for cytokines in serum specimens to all plasma specimens

Results show concentrations of analytes for all paired individuals (n=11) with the middle bar showing mean concentrations and bars the standard error of the mean.

A) Example of cytokines that yield higher measurements in serum than plasma

B) Example of cytokines that are not detectable in serum compared to plasma

C) Example of cytokines that yield lower measurements in serum than plasma

Figure 2. Example of cytokine concentrations in a serum specimen is similar to at least 1 type of plasma specimen

Results show concentrations as whisker box of analytes for all paired individuals (n=11) with median concentration shown by the line in the middle and the 1th and 99th percentile with the error bar. P values shown in the graph are calculated with non-parametric paired Wilcoxon test using serum specimens as controls.

- A) Eotaxin
- B) b-NGF
- C) SCGF-b

Figure 3. Example where measured concentrations of cytokines in plasma_{EDTA} is different from those in plasma_{citrate}

Results show concentrations as histograms of CTACK (A) and IP-10 (B) for all paired individuals (n=11) with error bar representing standard error of the mean. P values shown in the graph are calculated with non-parametric paired Wilcoxon test using plasma_{citrate} specimens as controls.

Figure 4. Normalization of measured concentrations of cytokines for Bio-rad kits
 Data were normalized in Bio-plex protocol manager software as Log2 (observed concentration) to normalize data, as $\log(\min) < \log(\text{mean}) < \log(\max)$.
 Arrows show cytokines below the detection limit, and yellow circles show 1 sample type that is different from the other 3, easily identifiable by the red/green colors.

Figure 5. Comparison of measured concentrations of eotaxin, IL-8 and IP-10 using kits from different vendors

Results are showing measured concentrations of eotaxin (A), IL-8 (B) and IP-10 (C) detecting in all 4 specimens type either with Bio-rad kits (left panel) or R&D kits (right panel).

Table 1

Characteristics of plasma/serum collection tubes.

Top color
Tube name	Yellow top	Lavender top	Green top	SST
Additive	Sodium Polyethanol Sulfonate (SPS) Acid citrate dextrose ACD solution A 22.0 g/L trisodium citrate 8.0 g/L citric acid 24.5 g/L dextrose solution B 13.2 g/L trisodium citrate 4.8 g/L citric acid 14.7 g/L dextrose	K2 EDTA (Ethylene Diamine Tetra-acetic Acid)	Sodium heparin Lithium heparin	Clot activator Silicone coated
Concentration of additive	0.109 mg/mL	1.5 mg/mL +/- 0.35 mg/mL	15 IU/mL +/- 2.5 IU/mL	0
Inversion needed	8	8	8	8
samples	plasma Citrate	plasma EDTA	Plasma Heparin	Serum

Summary of each collection method and additives.

Table 2

Mean concentrations of the 79 analytes

kit used	Analytes	values	Citrate	EDTA	Heparin	Serum
Human Cytokines Group -I (27-plex)	PDGF-bb pg/mL	mean	*195.4	*311.8	900.0	*9779.6
		% CV	129.4	109.0	51.5	32.1
		SEM	76.4	102.7	153.9	1000.7
	IL-1b pg/mL	mean	OOB<	OOB<	OOB<	OOB<
		% CV				
		SEM				
	IL-1ra pg/mL	mean	15.5	20.5	118.4	32.5
		% CV	90.3	84.9	307.0	73.8
		SEM	5.1	5.3	111.6	7.2
	IL-2 pg/mL	mean	OOB<	OOB<	OOB<	OOB<
		% CV				
		SEM				
	IL-4 pg/mL	mean	1.0	0.7	1.4	*4.6
		% CV	140.1	68.1	108.9	24.5
		SEM	0.5	0.2	0.5	0.4
	IL-5 pg/mL	mean	OOB<	OOB<	OOB<	OOB<
		% CV				
		SEM				
	IL-6 pg/mL	mean	5.9	1.9	2.8	2.3
		% CV	201.2	43.4	104.1	51.7
		SEM	4.4	0.2	0.9	0.3
	IL-7 pg/mL	mean	8.5	6.6	4.2	*10.1
		% CV	154.9	61.1	54.6	44.6
		SEM	4.3	1.3	0.7	1.4
	IL-8 pg/mL	mean	7.3	0.7	8.1	72.8
		% CV	178.2	52.8	109.1	176.6
		SEM	4.0	0.1	2.6	43.2
	IL-9 pg/mL	mean	2.2	2.2	5.3	14.1
		% CV	135.3	110.2	106.5	55.2

kit used	Analytes	values	Citrate	EDTA	Heparin	Serum
	IL-10 pg/mL	SEM mean % CV	3.9 OOR< OOR<	2.1 OOR< OOR<	2.4 OOR< OOR<	2.5 OOR< OOR<
	IL-12p70 pg/mL	SEM mean % CV	3.6 311.9 OOR<	1.0 108.1 OOR<	2.7 85.7 OOR<	6.6 55.6 OOR<
	IL-13 pg/mL	SEM mean % CV	4.8 OOR< OOR<	0.4 OOR< OOR<	0.8 OOR< OOR<	1.2 OOR< OOR<
	IL-15 pg/mL	SEM mean % CV	OOR< OOR< OOR<	OOR< OOR< OOR<	OOR< OOR< OOR<	OOR< OOR< OOR<
	IL-17 pg/mL	SEM mean % CV	7.2 136.9 OOR<	7.6 83.6 OOR<	42.0 212.2 OOR<	50.8 23.5 OOR<
	Eotaxin pg/mL	SEM mean % CV	3.3 44.4 56.9	1.9 27.9 52.5	29.0 49.9 53.8	3.8 62.7 48.8
	FGF basic pg/mL	SEM mean % CV	10.6 1.6 224.5	17.6 *0.2 170.6	9.4 1.8 120.2	19.4 *5.7 31.2
	G-CSF pg/mL	SEM mean % CV	1.3 5.0 60.8	0.1 7.6 81.4	0.8 41.2 196.0	0.6 5.5 62.1
	GM-CSF pg/mL	SEM mean % CV	1.6 OOR< OOR<	1.6 OOR< OOR<	29.7 OOR< OOR<	1.4 OOR< OOR<
	IFN-γ pg/mL	SEM mean % CV	19.6 178.2 13.2	11.7 68.7 2.5	33.8 151.7 17.9	52.6 101.3 15.1

kit used	Analytes	values	Citrate	EDTA	Heparin	Serum
	IP-10 pg/mL	mean	335.6	429.3	358.6	*480.7
		% CV	25.1	27.8	30.2	40.7
	MCP-1 pg/mL	SEM	25.4	36.0	33.8	61.2
		mean	*7.1	*4.6	16.0	11.9
	MIP-1a pg/mL	% CV	132.5	47.7	62.9	67.4
		SEM	2.9	0.6	3.4	2.4
	MIP-1b pg/mL	mean	3.5	2.1	3.9	6.2
		% CV	215.3	41.8	86.6	51.8
	RANTES ng/mL	SEM	2.3	0.3	1.0	1.0
		mean	*24.2	*24.0	36.7	51.5
	TNF-a pg/mL	% CV	34.9	27.3	36.9	59.9
		SEM	2.5	2.0	4.2	9.7
Human Diabeticis (10-plex)	C-peptide ng/mL	mean	*1.1	1.8	2.9	4.0
		% CV	39.9	54.4	48.6	50.4
	VEGF pg/mL	SEM	0.1	0.3	0.5	0.7
		mean	OOB<	OOB<	OOB<	OOB<
	Ghrelin pg/mL	% CV	9.7	*4.7	23.0	*76.9
		SEM	125.3	91.4	57.9	74.1
	GIP pg/mL	mean	3.9	1.5	4.4	18.6
		% CV	2.5	2.9	2.7	2.3
	GLP-1 pg/mL	SEM	49.7	72.2	54.0	68.6
		mean	0.4	0.6	0.4	0.5
		mean	715.2	1006.0	985.1	867.7
		% CV	35.1	41.9	57.2	35.3
	SEM	75.8	127.3	170.1	92.2	
	mean	313.2	371.2	365.2	171.6	
	% CV	74.3	88.1	63.1	67.0	
	SEM	70.3	98.8	69.6	34.7	
	mean	375.1	394.2	376.4	388.0	
	% CV	2.5	2.9	2.7	2.3	
	SEM	49.7	72.2	54.0	68.6	
	mean	0.4	0.6	0.4	0.5	
	mean	715.2	1006.0	985.1	867.7	
	% CV	35.1	41.9	57.2	35.3	
	SEM	75.8	127.3	170.1	92.2	
	mean	313.2	371.2	365.2	171.6	
	% CV	74.3	88.1	63.1	67.0	
	SEM	70.3	98.8	69.6	34.7	
	mean	375.1	394.2	376.4	388.0	
	% CV	2.5	2.9	2.7	2.3	
	SEM	49.7	72.2	54.0	68.6	
	mean	0.4	0.6	0.4	0.5	
	mean	715.2	1006.0	985.1	867.7	
	% CV	35.1	41.9	57.2	35.3	
	SEM	75.8	127.3	170.1	92.2	
	mean	313.2	371.2	365.2	171.6	
	% CV	74.3	88.1	63.1	67.0	
	SEM	70.3	98.8	69.6	34.7	
	mean	375.1	394.2	376.4	388.0	
	% CV	2.5	2.9	2.7	2.3	
	SEM	49.7	72.2	54.0	68.6	
	mean	0.4	0.6	0.4	0.5	
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	% CV	35.1	41.9	57.2	35.3	
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	% CV	74.3	88.1	63.1	67.0	
	SEM	70.3	98.8	69.6	34.7	
	mean	375.1	394.2	376.4	388.0	
	% CV	2.5	2.9	2.7	2.3	
	SEM	49.7	72.2	54.0	68.6	
	mean	0.4	0.6	0.4	0.5	
	mean	715.2	1006.0	985.1	867.7	
	% CV	35.1	41.9	57.2	35.3	
	SEM	75.8	127.3			

kit used	Analytes	values	Citrate	EDTA	Heparin	Serum
	Glucagon pg/mL	% CV	68.4	77.6	106.8	71.2
		SEM	77.4	92.2	126.9	83.3
		mean	157.2	155.1	123.4	128.6
	Insulin pg/mL	% CV	34.4	48.9	41.6	35.7
		SEM	15.7	22.9	16.1	14.4
		mean	419.3	662.1	644.7	505.6
	Leptin ng/mL	% CV	94.8	104.0	97.1	102.6
		SEM	120.0	208.2	189.0	157.5
		mean	5.6	7.4	8.7	6.8
	PAI-1 ng/mL	% CV	78.9	101.1	96.7	116.3
		SEM	1.3	2.3	2.5	2.4
		mean	*4.0	*3.9	8.5	*17.2
	Resistin ng/mL	% CV	31.0	28.1	31.7	40.9
		SEM	0.4	0.3	0.8	2.1
		mean	*3.7	*4.0	5.1	6.3
	Vifastin ng/mL	% CV	25.8	36.4	39.2	64.2
		SEM	0.3	0.4	0.6	1.2
		mean	0.7	0.8	1.6	2.0
Human Cytokines Group-II (21-plex)	CTAK ng/mL	% CV	126.3	89.5	120.6	115.6
		SEM	0.3	0.2	0.6	0.7
		mean	2.6	*3.3	2.8	*2.0
	GROa pg/mL	% CV	8.8	13.3	20.6	25.4
		SEM	0.1	0.1	0.2	0.2
		mean	56.3	43.4	47.9	95.8
	IL-1a pg/mL	% CV	80.8	54.2	50.9	63.5
		SEM	13.6	8.2	7.6	16.9
		mean	OOOR<	OOOR<	OOOR<	OOOR<
	IL2-ra pg/mL	% CV				
		SEM				
		mean	85.4	76.5	41.6	OOOR<
		% CV	57.2	72.0	75.3	

kit used	Analytes	values	Citrate	EDTA	Heparin	Serum
	IL-3 pg/mL	SEM	14.6	15.6	12.1	
		mean	153.3	148.3	116.7	OOOR<
		% CV	67.9	100.3	55.2	
	IL12p40 pg/mL	SEM	34.8	36.4	18.4	
		mean	303.5	519.3	272.3	161.5
		% CV	84.0	30.7	73.1	49.5
	IL-16 pg/mL	SEM	71.6	48.0	49.4	34.3
		mean	451.7	455.4	349.8	209.6
		% CV	21.3	28.3	36.1	78.8
	IL-18 pg/mL	SEM	29.0	38.9	41.7	51.1
		mean	40.2	46.4	63.0	26.8
		% CV	61.7	70.8	76.0	71.7
	LIF pg/mL	SEM	7.5	9.9	15.3	6.2
		mean	OOOR<	OOOR<	OOOR<	OOOR<
		% CV				
	MCP-3 pg/mL	SEM				
		mean	239.4	298.4	265.3	144.0
		% CV	30.6	41.5	38.0	32.9
	M-CSF pg/mL	SEM	21.9	40.6	37.1	19.8
		mean	OOOR<	OOOR<	OOOR<	OOOR<
		% CV				
	MIF pg/mL	SEM				
		mean	118.3	159.5	119.6	116.8
		% CV	43.3	36.0	54.8	105.4
	MIG pg/mL	SEM	15.4	17.9	21.3	46.4
		mean	683.9	823.2	997.9	602.1
		% CV	54.6	63.1	53.2	53.1
	b-NGF pg/mL	SEM	112.8	156.9	175.6	101.5
		mean	*9.6	*6.9	1.5	2.8
		% CV	45.9	67.8	76.5	110.4
		SEM	1.3	1.6	0.5	1.1

kit used	Analytes	values	Citrate	EDTA	Heparin	Serum
	SCF pg/mL	mean	*47.7	*55.3	87.1	*27.6
		% CV	33.7	43.5	48.0	67.2
		SEM	5.1	7.3	13.7	5.6
	SCGF-b ng/mL	mean	*14.0	*15.1	33.9	*13.6
		% CV	32.0	33.8	34.5	83.9
		SEM	1.4	1.5	3.9	3.6
	SDF-1a pg/mL	mean	76.2	83.5	55.1	OOOR<
		% CV	61.9	43.4	124.2	
		SEM	14.8	11.9	29.5	
	TNF-b pg/mL	mean	OOOR<	OOOR<	OOOR<	OOOR<
		% CV				
		SEM				
	TRAIL pg/mL	mean	124.6	106.6	115.4	93.9
		% CV	40.6	33.0	53.3	52.2
		SEM	15.3	11.7	20.3	16.7
	HGF pg/mL	mean	239.2	286.3	220.1	388.7
		% CV	37.3	35.0	51.3	68.0
		SEM	30.0	30.2	39.0	83.4
	IFN-a2 pg/mL	mean	112.0	138.0	109.8	87.6
		% CV	50.4	41.6	59.2	17.1
		SEM	16.6	16.0	18.2	7.0
Human Acute phase (5-plex)	PCT ng/mL	mean	6.6	7.8	7.2	*1.1
		% CV	39.4	22.4	30.1	77.6
		SEM	0.8	0.5	0.7	0.3
	Ferritin ng/mL	mean	165.4	210.0	233.9	134.0
		% CV	100.6	137.1	127.6	73.1
		SEM	50.3	87.1	90.3	29.6
	tPA ng/mL	mean	*11.8	8.8	5.7	10.9
		% CV	56.9	76.9	49.1	62.0
		SEM	2.1	2.0	0.8	2.0
	Fibrinogen µg/mL	mean	2000.2	4999.4	2510.6	*31.4

kit used	Analytes	values	Citrate	EDTA	Heparin	Serum
Human Acute phase (4-plex)	SAA ng/mL	% CV	56.9	50.9	66.1	318.4
		SEM	387.3	572.2	482.5	26.8
	SAA ng/mL	mean	9.2	9.3	9.7	10.0
		% CV	86.9	58.7	101.8	84.2
	SAA ng/mL	SEM	2.4	1.6	3.0	2.5
		A2M µg/mL	mean	1854.3	2264.9	1797.8
	% CV		38.8	44.6	46.2	46.8
	Haptoglobin µg/mL	SEM	218.1	305.7	251.4	271.2
		mean	809.8	698.6	654.1	531.2
	% CV	47.7	59.2	68.4	65.3	
Human Acute phase (4-plex)	CRP µg/mL	SEM	113.8	125.0	127.7	107.8
		mean	2.2	1.6	1.1	1.8
	% CV	183.2	155.4	215.4	188.8	
	SAP µg/mL	SEM	1.2	0.8	0.7	1.0
		mean	47.4	56.5	45.1	43.2
	% CV	61.7	64.7	83.9	47.9	
	SAP µg/mL	SEM	8.8	11.1	11.4	6.3
		CXCL5 pg/mL	mean	125.7	159.6	471.8
	% CV		83.58	96.43	74.81	75.27
	R&D (8-plex)	ITAC pg/mL	SEM	23.5	34.4	78.9
mean			17.44	19.36	284.7	36.57
% CV		58.05	61.36	68.24	49.93	
CCL-5 pg/mL		SEM	2.3	2.7	43.5	4.1
		mean	2758	5300	9019	13945
% CV		64.11	93.73	34.4	20.44	
CD40L pg/mL		SEM	395.5	1111.4	694.0	637.8
		mean	203.7	157.2	561.9	4960
% CV		69.82	44.31	49.82	69.36	
EGF pg/mL		SEM	31.8	15.6	62.6	769.6
	mean	8.505	7.284	10.95	64.92	

kit used	Analytes	values	Citrate	EDTA	Heparin	Serum
		% CV	79.25	90.78	65.74	139.62
		SEM	1.5	1.5	1.6	20.3
	<i>Eotaxin pg/mL</i>	<i>mean</i>	<i>77.1</i>	<i>59.1</i>	<i>844.7</i>	<i>119</i>
		% CV	<i>45.4</i>	<i>42.4</i>	<i>61.2</i>	<i>54</i>
		SEM	<i>7.8</i>	<i>5.6</i>	<i>115.6</i>	<i>14.4</i>
	<i>IL-8 pg/mL</i>	<i>mean</i>	<i>3.16</i>	<i>1.8</i>	<i>7.1</i>	<i>63.3</i>
		% CV	<i>123.8</i>	<i>87.1</i>	<i>70.1</i>	<i>232.1</i>
		SEM	<i>0.9</i>	<i>0.4</i>	<i>1.1</i>	<i>32.1</i>
	<i>IP-10 pg/mL</i>	<i>mean</i>	<i>23.9</i>	<i>28.8</i>	<i>37</i>	<i>7.5</i>
		% CV	<i>25.2</i>	<i>33.8</i>	<i>21.1</i>	<i>19.2</i>
		SEM	<i>1.4</i>	<i>2.2</i>	<i>1.8</i>	<i>0.3</i>

Results are shown as mean concentration, percentage of coefficient of variation and $\pm$ standard error of the mean for the analytes measured in plasma, citrate, plasma, EDTA, plasma, heparin and serum. Mann Whitney test was calculated using plasma, heparin as control, and significance is shown with * (set as <0.05).

Cytokines shown in italics for the R&D kits are the ones that are also detected with Bio-rad kits.

OOD = Out of range
%CV = percentage of variation of the mean.
SEM = standard error of the mean