Bio-Plex® suspension array system

tech note 5985

Development and Validation of Multiplex Assays for Human Diabetes Biomarkers

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Introduction

Diabetes and obesity have become major causes of morbidity and mortality in the United States, and their prevalence has increased steadily worldwide. As a result, there is a renewed focus on research toward prevention, early diagnosis, and treatment of diabetes. Profiling of multiple diabetes and metabolic biomarkers allows researchers to better understand the complex interactions among adipokines, gut hormones, and other biomolecules associated with diabetes and metabolic dysfunctions.

We have developed human diabetes multiplex immunoassays for simultaneous quantitation of diabetes and metabolic biomarkers in human serum and plasma of up to 12 targets, including adiponectin, adipsin, C-peptide, ghrelin, GIP, GLP-1, glucagon, insulin, leptin, PAI-1, resistin, and visfatin. Adiponectin and adipsin can be mixed as a 2-plex separately from the other targets due to their requirement for higher sample dilution, while the rest of the targets can be multiplexed to a 10-plex panel. These 10-plex targets can be further multiplexed with other cytokine biomarkers important in metabolism research and immune response, such as IL-6 and TNF-α. The diabetes immunoassays utilize the Bio-Plex® suspension array system and Bio-Plex Pro™ magnetic COOH beads on a 96-well platform, with the option of using the Bio-Plex Pro wash station for automated bead washing. The assays allow for analysis of multiple biomarkers in less than 3 hr with as little as 12.5 µl of serum or plasma samples per well. Performance characteristics, including sensitivity, specificity, working ranges, precision, accuracy, and linearity of dilutions in both serum and plasma were validated. The assays were further evaluated with normal and diabetes serum and plasma samples. These Bio-Plex immunoassays are shown to be both sensitive and precise, and are ideal for clinical research and high-throughput screening for diabetes and metabolic biomarkers.

Methods

Experimental procedures for the assay were carried out at ambient temperature. Briefly, the antibody-coupled beads were first incubated with antigen standards or samples for 1 hr. They were then washed using the Bio-Plex Pro wash station or a vacuum filter to remove unbound materials, then incubated with biotinylated detection antibodies for 30 min. After washing away the unbound biotinylated antibodies, the beads were incubated with SA-PE for 10 min. Following removal of excess SA-PE, the beads were passed through the Bio-Plex array reader, which measures the fluorescence of the bound SA-PE. Data analysis was performed using Bio-Plex Manager™ software version 6.0, with the photomultiplier tube set at high.

Results

Assay Sensitivity, Precision, Accuracy, and Working Ranges

Assay sensitivity (limit of detection, LOD) was calculated as the concentration of analyte for which the corresponding fluorescence signal is two standard deviations above the background measured in the blank. Intra-assay precision was calculated as the coefficient of variation (%CV) among fluorescence values of replicate wells on a representative assay plate. Inter-assay precision was calculated as the %CV of the observed concentrations of target antigen from independent assays. Assay accuracy (recovery) was calculated as the percentage of the observed concentration value of a target antigen relative to the expected value. Assay working range is the range of concentrations in which the assay is both precise (intra- and inter-assay CV < 20%) and accurate (80–120% recovery), and is represented by lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ). The assay working ranges, LOD, and intra- and inter-assay %CV obtained with a serum-based matrix for all targets of the Bio-Plex Pro human diabetes panel are summarized in Table 1. Similar results were observed for assays in RPMI cell culture medium matrix.

Table 1: Assay working ranges, sensitivity, and precision. The LLOQ, ULOQ, LOD and inter-assay %CV are mean data determined from three independent multiplex assays in serum-based matrix. Intra-assay %CV is derived from one representative assay. LLOQ and ULOQ are defined as the boundary standard curve points within which the performance specifications of each standard point were met for 10% intra-assay CV, 15% inter-assay CV, and recovery range of 80–120%. Exceptions are noted for GLP-1 with one point exhibiting 12% intra-assay CV, and for leptin with one point exhibiting 17% inter-assay CV. Data were generated using the magnetic workflow with the Bio-Plex Pro II wash station.

	Working Range, pg/ml		Sensitivity, pg/ml	Precision	
Target	LLOQ	ULOQ	LOD	Intra-Assay %CV	Inter-Assay %CV
2-plex assays					
Adiponectin	160	218,485	32.7	4	2
Adipsin	43	14,513	17	6	4
10-plex assays					
C-peptide	22.4	10,031	14.5	5	4
Ghrelin	16.6	8,502	1.2	4	2
GIP	11.2	22,895	0.8	3	4
GLP-1	31.3	16,000	5.3	6	3
Glucagon	15.7	3,500	4.9	5	6
Insulin	1.7	3,541	1	3	5
Leptin	11.5	129,107	3.1	3	4
PAI-1	8.8	47,850	2.2	5	4
Resistin	2.3	4,739	1.3	3	4
Visfatin	51.3	280,266	37.1	4	3

Assay Specificity (% Cross Reactivity)

Assay specificity was examined by performing assays with multiplex beads and multiplex antigen standards at the fourth dilution point of the standard curve. Detection antibodies were added individually. Cross reactivity was defined as the percentage of nonspecific, cross-reacting signal detected relative to the specific signal for that analyte. No cross reactivity was observed among the 10-plex assays, as shown in Table 2. No cross reactivity was observed between adiponectin and adipsin either.

Assay Linearity of Dilution

Linearity of dilution ensures that analytes present in concentrations above the LLOQ can be diluted and measured accurately within the assay working ranges. Depending on the endogenous levels of analytes, linearity of dilution was examined by either diluting serum or plasma samples directly with standard diluent or diluting spiked samples with human serum or plasma in 1:3 serial dilutions. The observed and expected sample concentrations within the assay working range were plotted for each analyte. The correlation coefficient R^2 value generated by linear regression analysis reflects the linearity of dilution for that assay. The R^2 values are 0.98 or higher for all targets in the diabetes panel. Figure 1 is a representative linearity of dilution plot for ghrelin assay in serum matrix.

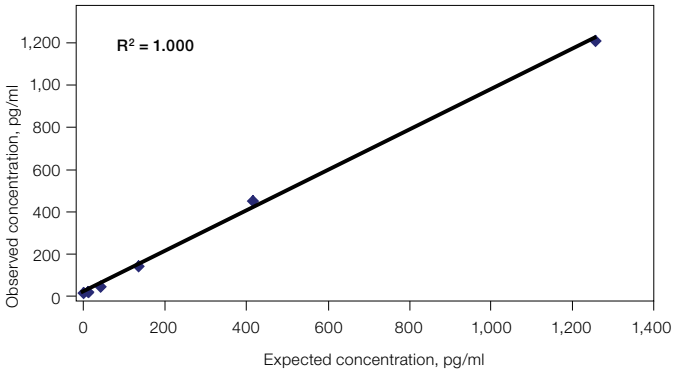

Fig. 1. Linearity of dilution plot for ghrelin assay in human serum. Spiked serum sample was diluted four-fold (1:3) with human serum. The observed and expected sample concentrations within the assay working range were plotted, and the correlation coefficient R^2 value was generated by linear regression analysis.

Table 2. Assay specificity (% cross reactivity). Percentage of cross reactivity was calculated based on fluorescence signal detected at the fourth dilution point of the standard curve using multiplexed capture beads and multiplexed antigens in the presence of a single detection antibody.

Target	C-peptide	Ghrelin	GIP	GLP-1	Glucagon	Insulin	Leptin	PAI-1	Resistin	Visfatin
C-peptide		0.0	0.1	0.1	0.0	0.0	0.0	0.0	0.2	0.5
Ghrelin	0.2		0.0	0.1	0.0	0.0	0.1	0.2	0.1	0.3
GIP	0.4	0.1		0.1	0.1	0.0	0.0	0.1	0.1	0.0
GLP-1	0.5	0.0	0.6		0.1	0.1	0.1	0.3	0.0	0.5
Glucagon	0.3	0.1	0.2	0.4		0.0	0.0	0.0	0.0	0.1
Insulin	0.2	0.0	0.3	0.2	0.1		0.1	0.2	0.0	0.0
Leptin	0.0	0.0	0.0	0.0	0.1	0.1		0.2	0.0	0.2
PAI-1	0.2	0.0	0.0	0.1	0.1	0.0	0.0		0.1	0.1
Resistin	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.6		0.0
Visfatin	0.1	0.0	0.2	0.0	0.0	0.0	0.0	0.1	0.1	

Assay Parallelism

Assay parallelism is another way of examining matrix effect on assay performance. This was investigated by comparing the slope of the spike concentration-response curve in human serum or plasma with that of the standard curve in standard diluent. The similarity between the curve slopes demonstrates assay parallelism between standard diluent and human serum or plasma. The assays of high endogenous analytes do not exhibit a valid response curve with antigen spikes in human serum or plasma. Therefore, parallelism data are available only for those analytes with relatively low endogenous levels, such as C-peptide, ghrelin, GIP, GLP-1, glucagon, and insulin. The percentage differences between the slopes of the concentration-response curve in human serum or plasma and the slope of the standard curve are in the range of 0.9–23% for these targets, except for C-peptide in plasma, which is at 32%. Figure 2 shows the parallelism of the spike concentration-response curve in plasma and the standard curve for glucagon.

Analysis of Human Samples

A total of ten normal and 28 type II diabetes serum samples were examined with the Bio-Plex Pro human diabetes panel. Data were analyzed using Bio-Plex Manager software version 6.0. Recovery range specification was set at 80–120%. As shown in Figure 3 for insulin, the assay working range spans the measured concentration ranges of the majority of samples analyzed. Statistical analysis was performed using Student's *t*-test, and a *P* value of less than 0.05 was considered significant. As shown in Figure 4 and Table 3, type II diabetes samples showed significant elevation of adipsin, ghrelin, GLP-1, glucagon, insulin, PAI-1, and resistin.

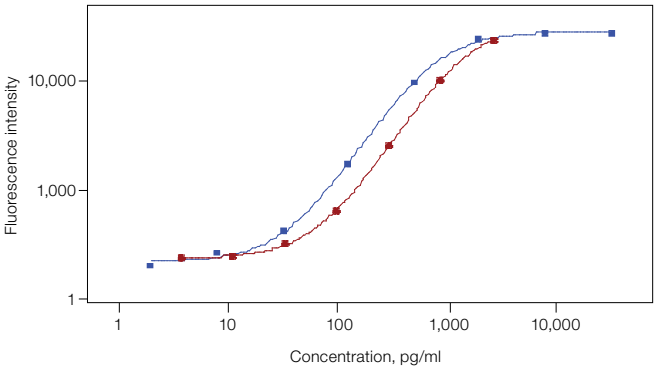

Fig. 2. Assay parallelism for glucagon in human plasma. A 7-point spike concentration-response curve in plasma and an 8-point standard curve in standard diluent were compared with 4-PL curve fitting. The slope difference between these two curves is 0.9%. ■, standard curve in standard diluent; ■, spike concentration-response curve in human plasma.

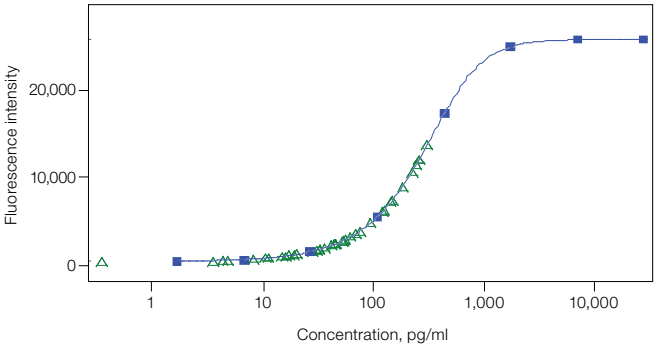

Fig. 3. Standard curve with samples for insulin. Assay working range spans the measured concentration ranges of a typical set of unknown serum samples. Results for other assays were similar. ■, standards; Δ, unknowns.

Table 3. Levels of diabetes and metabolic biomarkers in normal and type II diabetes serum samples.

	Levels of Diabetes/Metabolic Biomarkers in Human Serum Samples, pg/ml			
	Normal, n = 10		Type II Diabetic, n = 28	
Analyte	Range, min–max	Mean ± SD	Range, min–max	Mean ± SD
Adiponection	747,590–9,098,600	2,931,709 ± 2,393,052	620,283–15,145,000	4,395,835 ± 3,699,703
Adipsin	268,482–556,739	425,867 ± 84,594	219,772–1,127,400	616,374 ± 248,281*
C-peptide	137–1,220	475 ± 322	197–3,034	888 ± 735
Ghrelin	0–58	19 ± 21	24–1,422	350 ± 313*
GIP	0–163	49 ± 56	44–2,836	296 ± 517
GLP-1	0–129	20 ± 41	71–456	211 ± 102*
Glucagon	0–82	14 ± 26	78–457	184 ± 97*
Insulin	1–506	108 ± 154	43–1,260	391 ± 365*
Leptin	22–5,184	918 ± 1,778	130–42,208	4,750 ± 8,031
PAI-1	6,019–20,539	9,705 ± 4,343	1,842–45,173	20,786 ± 10,348*
Resistin	555–3,652	1,816 ± 893	453–22,253	5,271 ± 5,145*
Visfatin	0–1,230	228 ± 402	5–35,857	3,562 ± 6,697

* *P* <0.05 with Student's *t*-test.

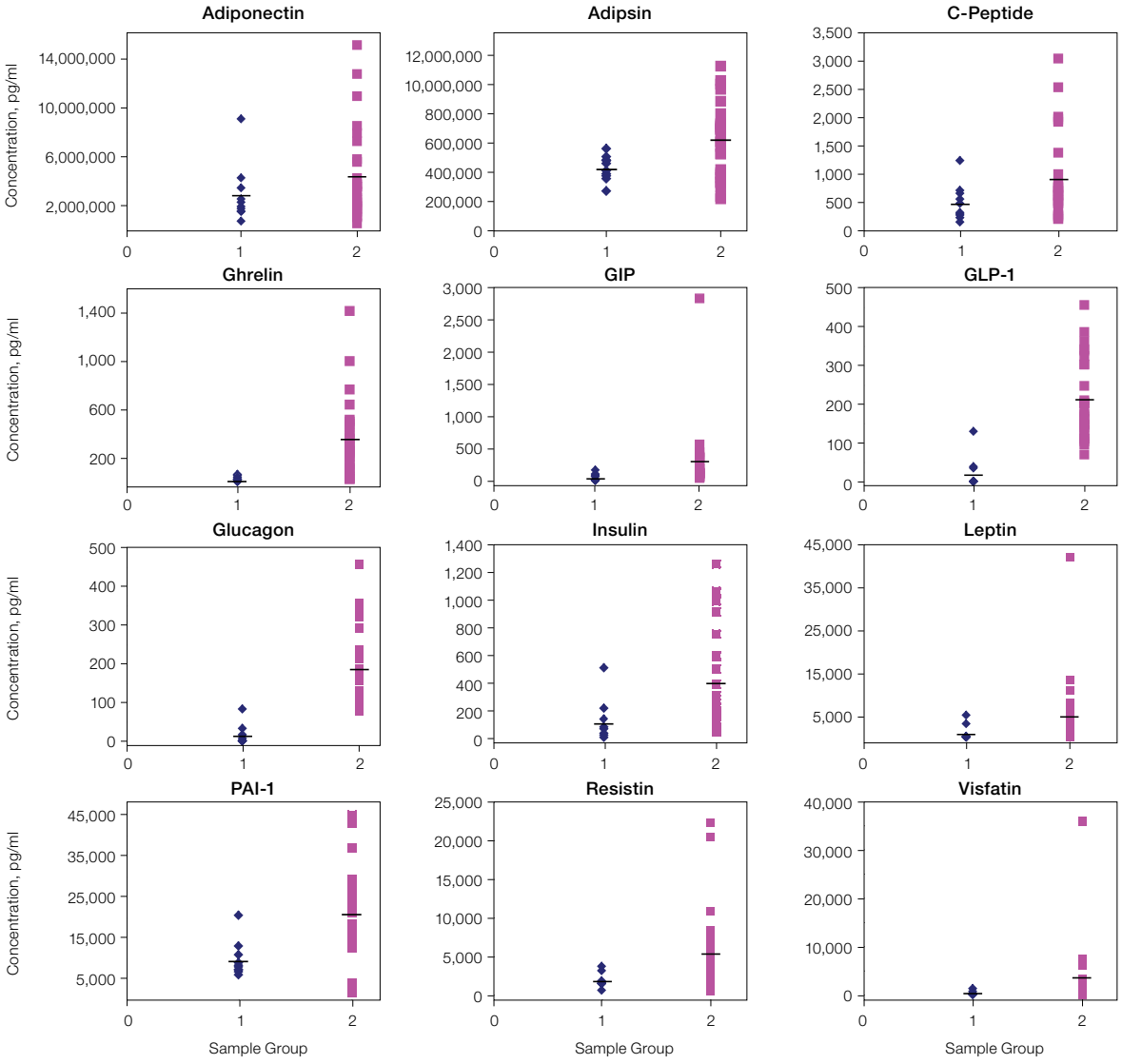

Fig. 4. Analysis of normal and type II diabetes serum samples with the Bio-Plex Pro human diabetes panel. Data were analyzed with Bio-Plex Manager software version 6.0. The mean for each group of samples is marked with a black line. Statistical analysis was performed using Student's *t*-test. Type II diabetes samples showed significant elevation of adipsin, ghrelin, GLP-1, glucagon, insulin, PAI-1, and resistin (P <0.05). ◆, normal; ■, type II diabetic.

Conclusion

The magnetic bead-based Bio-Plex Pro diabetes panel allows simultaneous measurement of multiple diabetic and metabolic biomarkers in a single sample in serum, plasma, and cell culture medium matrices. The implementation of magnetic bead–based assays allows for automation of assay wash steps (using the Bio-Plex Pro wash station) or the complete robotic automation of assay processing. The assays have been shown to achieve technical specifications in sensitivity, specificity, precision, accuracy, assay working ranges, linearity of dilution, and parallelism. Human sample analysis has demonstrated the validity of these assays; the analyte concentration ranges of a typical sample set are mostly within assay working ranges. The results are also consistent with published reports that levels of most of these biomarkers increase in diabetic conditions.

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