Mouse Diabetes Multiplex Metabolic Biomarkers Assay

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Abstract

More than 220 million people worldwide suffer from diabetes, a disorder that can lead to a series of complications and metabolic disorders arising from interactions among multiple proteins. The obese/diabetic mouse serves as an excellent model for the study of diabetes and its metabolic implications for drug discovery and targeted therapies. We have developed a novel mouse 8-plex immunoassay to measure a selection of common metabolic biomarkers (ghrelin, GIP, GLP-1, glucagon, insulin, leptin, PAI-1, resistin) and a singleplex immunoassay for adiponectin. These metabolic biomarker immunoassays may be multiplexed with Bio-Plex Pro™ cytokine assays such as IL-6 and TNF-α to extend the biomarker profile. The assays are based on Bio-Plex Pro magnetic COOH beads, which allow the implementation of automated wash steps using the Bio-Plex Pro wash station to improve efficiency and precision. The performance of these mouse metabolic biomarker assays was evaluated for specificity, sensitivity, precision, and accuracy. In addition, linearity of these biomarkers was demonstrated in mouse serum, plasma, and cell culture medium. Overall, these Bio-Plex assays were shown to be highly specific, accurate, and precise.

Introduction

Diabetes mellitus, often simply referred to as diabetes, is a condition in which a person has high blood sugar (glucose) levels as a result of the body either not producing enough insulin or because cells do not properly respond to the insulin that is produced. Insulin, a hormone produced in the pancreas, enables cells to absorb glucose to create energy. If the body's cells do not absorb the glucose, it accumulates in the blood; this condition is known as hyperglycemia. This leads to various potential medical complications. The two most common types of diabetes are type I and type II diabetes. Type I diabetes results from the body's failure to produce insulin and requires insulin injections. Type II diabetes is a condition in which cells fail to use insulin properly and may be combined with an absolute insulin deficiency. Type II diabetes is the most prevalent condition, affecting 90–95% of the diabetic population, and is closely associated with obesity. Obese mouse models that develop diabetes are widely used to study this disease in animals; however, sample volume is limiting in these cases. We have developed Bio-Plex Pro mouse diabetes assays using Luminex xMAP technology to measure nine biological markers for diabetes research in a single sample.

Methods

Performance of the Bio-Plex Pro mouse diabetes 8-plex assay and adiponectin assay was evaluated using serum- or cell culture media–based standard diluent and mouse serum or plasma samples. Experimental procedures for the assay were carried out at room temperature according to the manufacturer's instructions (Bio-Rad bulletin 10010747). Briefly, the antibody-coupled beads were first incubated with antigen standards or samples for 1 hr. Samples were diluted fourfold in sample diluent for the 8-plex assay; adiponectin was evaluated as a single assay due to the higher sample dilution requirement (1:1,600). The beads were then washed using the Bio-Plex Pro wash station to remove unbound materials and subsequently incubated with biotinylated detection antibodies for 30 min. The beads were then 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, at a high PMT (photomultipler tube) setting. Data analysis was performed using Bio-Plex Manager™ software version 6.0.

Results

Major performance parameters for Bio-Plex Pro mouse diabetes assays were verified in at least three independent assays. The parameters were specificity, sensitivity, accuracy, and precision. Standard response curves of all nine analytes are shown in Figure 1. Tables 1, 2, and 3 summarize assay specificity, sensitivity, working ranges, and precision, respectively.

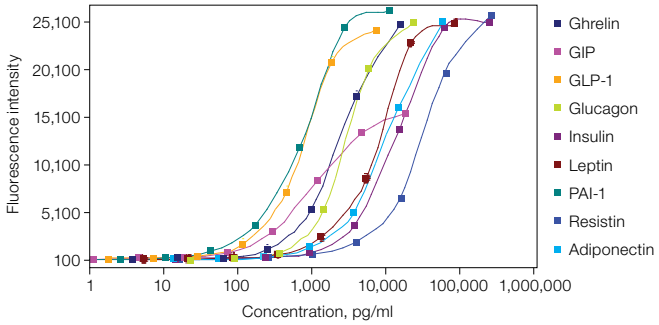

Fig. 1. Standard curves for Bio-Plex Pro mouse diabetes assays. The standard analyte concentrations vary according to different physiological biomarker levels.

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Table 1. Assay specificity reflected by cross reactivity (%) among the targets in the Bio-Plex Pro mouse diabetes panel. Percentage of cross reactivity was calculated using the median fluorescence intensity (MFI) of multiplexed detection antibodies and capture antibody–coupled beads in the presence of a single antigen at the third highest concentration in the standard curve.

Target	Adiponectin	Ghrelin	GIP	GLP-1	Glucagon	Insulin	Leptin	PAI-1	Resistin
Adiponectin		–0.1	–0.3	–0.4	–0.2	–0.4	–0.2	–0.1	–0.2
Ghrelin	0.0		–0.2	–0.3	–0.2	–0.1	–0.1	0.0	–0.4
GIP	–0.1	–0.1		1.4	–0.2	0.2	0.0	0.0	–0.2
GLP-1	0.0	–0.1	–0.3		–0.3	–0.1	0.1	0.0	–0.2
Glucagon	–0.1	–0.1	–0.4	–0.7		–0.1	–0.1	0.0	–0.2
Insulin	0.0	–0.1	–0.4	–0.9	–0.3		–0.1	0.0	–0.3
Leptin	0.0	–0.1	–0.3	–1.2	–0.4	–0.2		0.0	–0.4
PAI-1	0.1	0.0	–0.1	–0.7	–0.3	0.0	0.2		0.1
Resistin	0.0	0.0	0.0	0.0	0.0	–0.7	0.0	0.0	

Assay Specificity (% Cross Reactivity)

Assay specificity was examined by performing assays with multiplexed capture antibody–coupled beads and detection antibodies in the presence of a single antigen at the third highest concentration in the standard curve. Cross reactivity was defined as the percentage of nonspecific, cross-reacting signal detected relative to the specific signal for that analyte. Data demonstrate that cross reactivity among the nine targets is negligible.

Assay Sensitivity, Accuracy, Working Ranges, and Precision

Assay sensitivity, also known as the lower limit of detection (LOD), is defined as the concentration of an analyte obtained at the MFI that is two standard deviations above the background measured in the blank. Assay accuracy (recovery) was calculated as the percentage of the observed concentration value of a target antigen relative to the expected value. The lower and upper limits of quantitation (LLOQ and ULOQ, respectively) 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%. Mean LOD, LLOQ, and ULOQ of three independent assays are shown in Table 2. The LOD for all nine targets was sufficient to effectively measure analytes in normal and diabetic biological samples. The working assay ranges allowed accurate and precise measurement of analytes from normal and diabetic mouse samples.

Table 2. Assay sensitivity and working ranges of the Bio-Plex Pro mouse diabetes assay.

	Concentration, pg/ml					
	Serum-Based Matrix			Cell Culture Medium		
Target	LOD	LLOQ	ULOQ	LOD	LLOQ	ULOQ
Adiponectin*	8.4	15.0	63,391	0.4	15.0	62,043
Ghrelin	0.8	3.1	8,338	37.0	260.0	16,676
GIP	2.3	3.7	19,999	8.3	4.9	19,999
GLP-1	0.8	1.4	1,969	4.6	1.9	7,877
Glucagon	7.0	6.0	3,066	11.0	96.0	6,133
Insulin	22.0	93.0	31,876	53.0	62.0	63,753
Leptin	6.2	17.1	69,900	14.0	22.8	93,200
PAI-1	0.5	0.7	2,921	0.8	0.7	2,921
Resistin	32.0	63.0	257,870	54.0	63.0	257,870

* Adiponectin is a singleplex assay.

Intra-assay precision was determined from the variance of MFI of three replicate wells for eight standard points within the assay range. The mean intra-assay %CV is shown from one representative assay. The inter-assay precision was measured from the variance of observed concentrations of eight standard points for three independent assays. The mean intra- and inter-assay %CV for standard points within the assay range are shown in Table 3.

Table 3. Intra- and inter-assay %CV.

	Assay Precision	
Target	Intra-Assay %CV	Inter-Assay %CV
Adiponectin	4	3
Ghrelin	5	4
GIP	4	10
GLP-1	6	11
Glucagon	6	6
Insulin	6	4
Leptin	4	3
PAI-1	5	2
Resistin	4	4

Linearity of Dilution and Parallelism

Linearity of dilution ensures that analytes present in concentrations within the assay range can be diluted and measured accurately for relative quantitation. Linearity was demonstrated in mouse serum, plasma, and cell culture medium with R^2 >0.99 for all nine targets (Table 4).

Table 4. Linearity of dilution.

Linearity of Sample Dilutions (R^2)*	Serum	Plasma	RPMI-10% FCS
Adiponectin	0.9941	0.9929	0.9992
Ghrelin	0.9972	0.9996	0.9972
GIP	0.9998	0.9986	0.9998
GLP-1	1.0000	0.9993	1.0000
Glucagon	0.9999	0.9939	0.9927
Insulin	0.9999	0.9998	0.9999
Leptin	0.9989	0.9990	0.9989
PAI-1	0.9985	0.9987	0.9985
Resistin	0.9932	0.9998	0.9989

*The correlation coefficient (R^2) was determined by linear regression analysis of analytes measured in threefold serial dilutions of standard-spiked samples within assay range.

Examining parallelism is another way to determine matrix effects on assay performance. Parallelism was demonstrated between spiked samples and standards with 4-PL curve slope differences <18% for all nine targets measured within assay range. Using glucagon as an example (Figure 2), the similarity between the curve slopes demonstrates assay parallelism.

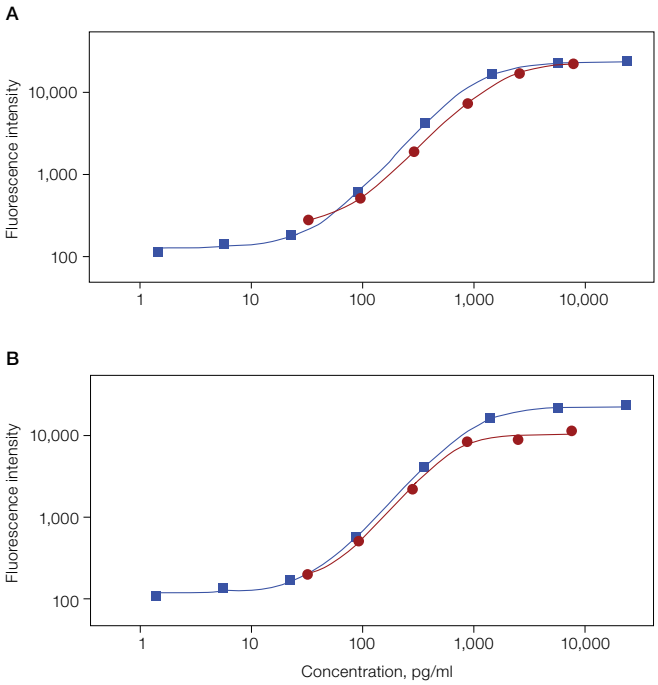

Fig. 2. Parallelism for glucagon. Examples of parallelism in mouse serum (**A**) and plasma (**B**) between spiked samples (●) and standard curves (■). Curves were fitted with 4-PL regression analysis.

Sample Measurements

Native antigen detection in biological sample matrices was verified by testing a total of 68 representative mouse samples (n = 28 normal; n = 40 diabetic) in the nine mouse assays. All assays were able to detect the majority of samples (68–100% normal; 75–100% diabetic) within the working range of each assay. A sample population of normal (n = 16) and diabetic (n = 23) mouse serum and plasma target values is shown in Figure 3, demonstrating that target values fall within the accurate range of the 5-PL fit standard curve.

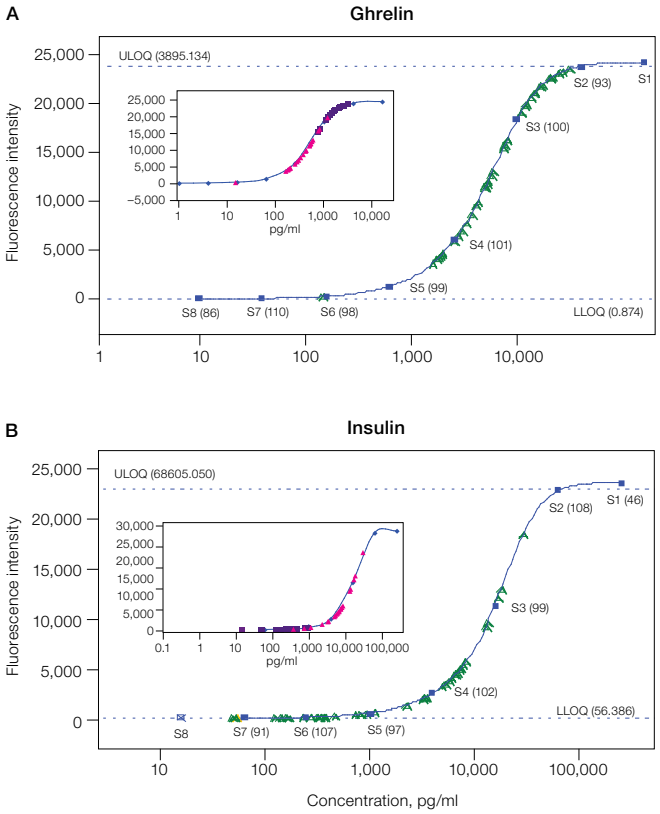

Fig. 3. Normal and diabetic mouse ghrelin (A) and insulin (B) levels fall within working assay range. Assay working range (80–120% standard recovery, 10% intra-assay CV) spans the measured concentration ranges of a typical set of unknown samples including fasting and fed animal samples. Results for other assays were similar. ■, standards; △, samples. Insets: ■, normal samples; ▲, diabetic samples.

As shown in Table 5 and Figure 4, statistical significance was established between normal and diabetic mouse samples for known diabetic biomarkers.

Table 5. Levels of diabetes and metabolic biomarkers in normal and diabetic mouse serum and plasma samples.

	Normal, n = 16**		Diabetic, n = 23**	
Analyte	**Range**	**Mean ± SD**	**Range**	**Mean ± SD**
Adiponectin	5,361,480–6,114,980	12,048,003 ± 4,957,426	16,056,810–23,458,845	19,927,746 ± 3,176,442*
Ghrelin	3,079–12,823	6,940 ± 2,777	60–4,874	1,685 ± 1,077*
GIP	26–187	85 ± 52	27–918	164 ± 190
GLP-1	6–99	40 ± 31	6–45	20 ± 10*
Glucagon	62–491	205 ± 127	109–678	266 ± 162
Insulin	501–3,697	1,375 ± 1,017	1,493–119,328	31,402 ± 27,683*
Leptin	46–1,712	637 ± 571	4,435–84,586	40,610 ± 22,240*
PAI-1	1,288–12,144	3,048 ± 2,558	171–6,919	2,443 ± 1,879
Resistin	106,145–247,535	160,585 ± 45,581	14,233–598,863	244,158 ± 196,688

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

**In the adiponectin assay n = 4 for normal samples and n = 5 for diabetic samples.

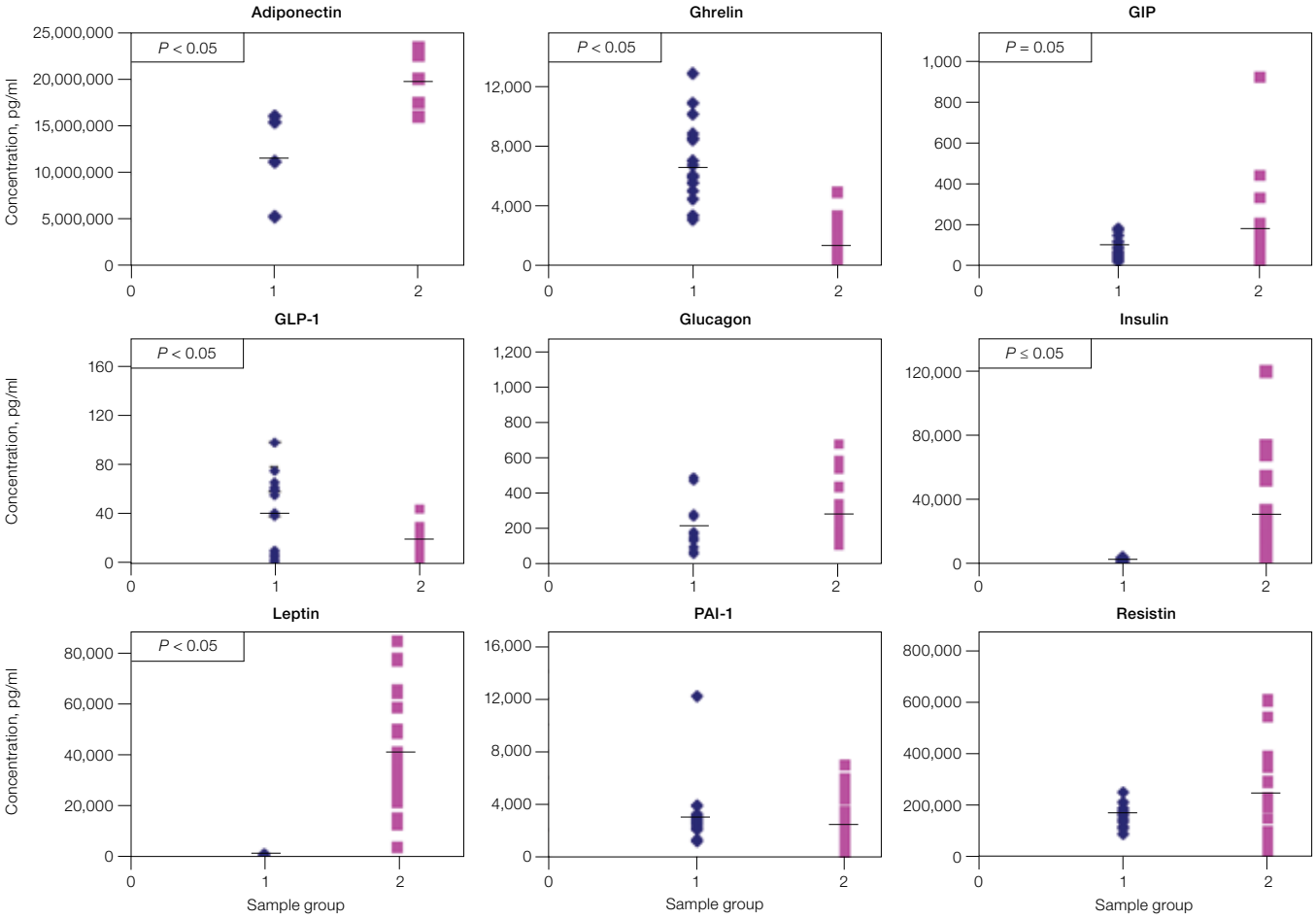

Fig. 4. Dot plot analysis of normal (n = 16) and diabetic (n = 23) mouse serum and plasma samples. One representative assay consisted of 39 mouse samples. The mean biomarker level for each group is marked with a black line. A Student's *t*-test established statistical significance between normal (◆) and type II diabetic (■) groups ($P \leq 0.05$) for established biomarkers.

Conclusions

We have developed novel mouse diabetes assays: the Bio-Plex Pro mouse diabetes 8-plex assay and Bio-Plex Pro adiponectin assay. The assays have been optimized for high performance (specificity, sensitivity, accuracy, and precision) in conjunction with sample linearity and a broad assay range to measure a wide population of mouse samples as required for preclinical research applications.

The Bio-Plex Pro mouse diabetes assays, which were developed on magnetic beads, are compatible with the automatic Bio-Plex Pro wash station for better precision and ease of workflow. Additionally, these assays can be multiplexed with the Bio-Plex Pro mouse cytokine and growth factor assays (23-plex and 9-plex panels) for custom assay blends to enhance the biomarker profile studied.

The development and verification of these assays represent significant progress in improving the multiplex assay quality and versatility required for diabetes research.

Information in this tech note was current as of the date of writing (2010) and not necessarily the date this version (rev A, 2010) was published.

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