Mouse Diabetes Multiplex Metabolic Biomarkers Assay

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Abstract

More than 220 million people worldwide suffer from diabetes. Diabetes leads to a series of complications and metabolic disorders that arise from interactions among proteins. The obese/diabetic mouse serves as an excellent model to study diabetes and its metabolic implications for drug discovery and targeted therapies. Bio-Rad has developed a novel mouse 8-plex immunoassay to measure the metabolic biomarkers (ghrelin, GIP, GLP-1, glucagon, insulin, leptin, PAI-1, resistin) and a single-plex immunoassay for adiponectin, which requires a higher sample dilution due to higher physiological levels. These metabolic biomarker immunoassays may be multiplexed with similar immunoassays for cytokines, i.e. IL-6 and TNFα. These assays utilize Bio-Plex Pro™ magnetic COOH beads that allow the option of implementing automated wash steps using the Bio-Plex Pro wash station. The performance of these mouse metabolic biomarker assays was evaluated for sensitivity, specificity, precision, and accuracy. In addition, linearity of these biomarkers was demonstrated in mouse serum, plasma, and cell culture matrix. 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 body cells do not properly respond to the insulin that is produced. Insulin is a hormone produced in the pancreas that enables body cells to absorb glucose to turn into energy. If the body's cells do not absorb the glucose, it accumulates in the blood (hyperglycemia), leading to various potential medical complications. There are many types of diabetes, the most common of which are type I diabetes, which results from the body's failure to produce insulin and requires insulin injections, and type II diabetes, which results from insulin resistance, a condition in which cells fail to use insulin properly and which is sometimes 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. Bio-Rad Laboratories has developed Bio-Plex Pro mouse diabetes assays using Luminex xMAP technology to measure nine biological markers for diabetes research.

Methods

The performance of the Bio-Plex Pro mouse diabetes 8-plex assay kit (Figure 1) and adiponectin assay kit were evaluated using serum- or cell culture media–based standard diluent and mouse serum or plasma samples. Samples were diluted four-fold in sample diluent for the 8-plex assays, whereas adiponectin was evaluated as a single assay due to the requirement of a higher sample dilution (1:1,600). Figure 2 illustrates a schematic representation of a multiplex bead-based sandwich immunoassay on the Bio-Plex suspension array system.

Fig. 1. Bio-Plex Pro mouse diabetes 8-plex assay kit (catalog #171-F7001M).

The Bio-Plex suspension array system integrates a series of color-coded magnetic beads (Bio-Plex Pro magnetic COOH beads), each of which is coupled to a unique antibody specific for a select biochemical marker. Beads are dyed with differing concentrations of two fluorophores to generate distinct bead sets. Each bead set is coated with a capture antibody specific for one analyte. Captured analyte is detected using a biotinylated detection antibody and streptavidin-phycoerythrin (SA-PE). Following each incubation period the capture bead complex is washed using the Bio-Plex Pro wash station and Bio-Plex Pro flat-bottom 96-well plate. Analysis is performed on a Bio-Plex 200 system with high throughput fluidics (HTF), which is a dual laser, flow-based sorting and detection platform. One laser is bead-specific and determines which diabetes biomarker is being detected. The other laser determines the magnitude of PE-derived signal, which is in direct proportion to the amount of analyte bound.

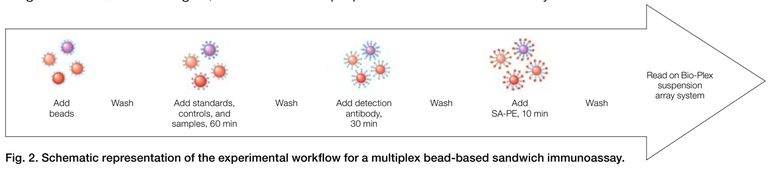


Fig. 2. Schematic representation of the experimental workflow for a multiplex bead-based sandwich immunoassay.

Results

Major performance parameters for Bio-Plex Pro mouse diabetes assays were verified in at least three independent assays. Standard response curves of all nine analytes are shown in Figure 3. Tables 1, 2, and 3 summarize assay specificity, sensitivity, working ranges, and precision, respectively. Data demonstrate that cross reactivity among the eight targets is negligible. The lower limit of detection (LOD) for all nine targets is sufficient to effectively measure analytes in normal and diabetic biological samples. The working assay ranges allowed for accurate and precise measurement of analytes for a majority of normal and diabetic mouse samples. Linearity (R^2 >0.99) and parallelism are demonstrated for all nine targets in three matrices: mouse serum, plasma, and cell culture medium.

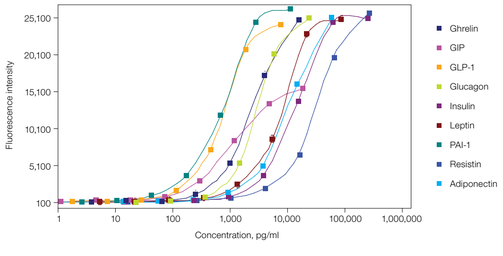


Fig. 3. Standard curves for Bio-Plex Pro mouse diabetes assays. The standard analyte concentrations vary according to different physiological biomarker levels.

Assay Specificity

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 Sensitivity

Table 2. Assay sensitivity and working ranges of the Bio-Plex Pro mouse diabetes assay. The lower limit of detection (LOD) is defined as the concentration obtained at the MFI that is two standard deviations above background. The lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) values define the assay working range in which the assay is highly accurate (80–120% recovery) and precise (≤10% intra-assay CV). Mean LOD, LLOQ, and ULOQ of 3 independent assays are shown below.

	Concentration, pg/ml					
	Serum-based Matrix			Cell Culture Medium		
Target	LOD	LLOQ	ULOQ	LOD	LLOQ	ULOQ
Adiponectin*	8.4	15	63,391	0.4	15	62,043
Ghrelin	0.8	3.1	8,338	37	260	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	6	3,066	11	96	6,133
Insulin	22	93	31,876	53	62	63,753
Leptin	6.2	17.1	69,900	14	22.8	93,200
PAI-1	0.5	0.7	2,921	0.8	0.7	2,921
Resistin	32	63	257,870	54	63	257,870

* Adiponectin is a singleplex assay.

Assay Precision

Intra-assay precision was determined from the variance of MFI of three replicate wells for eight standard points. The mean intra-assay %CV of standard points within assay range 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 inter-assay %CV of standard points within assay range (80–120% recovery) is shown (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 media with R^2 >0.99 for all nine targets (Table 4). Parallelism is necessary to reveal similarly behaving sample matrix effects. Parallelism was demonstrated between spiked samples and standards with 4-PL curve slope differences <18% for all nine targets measured within assay range (Figure 4).

Table 4. Linearity of dilution. The correlation coefficient (R^2) was determined by linear regression analysis of analytes measured in three-fold serial dilutions of standard-spiked samples within assay range.

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

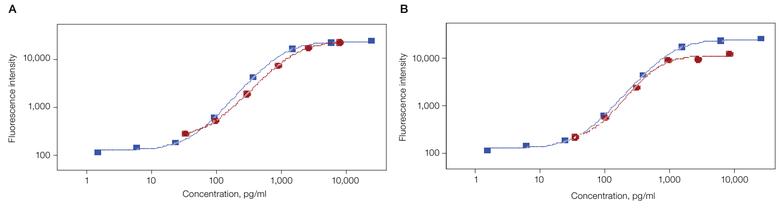
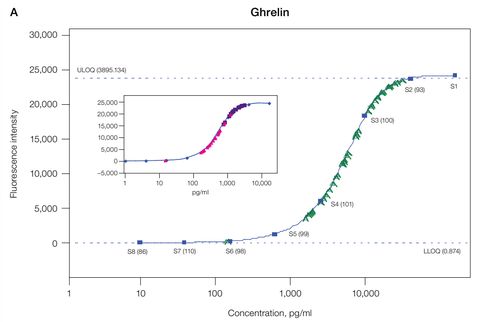


Fig. 4. Parallelism for glucagon. Examples of parallelism in mouse serum (A) and plasma (B) between spiked samples (■) and standard curves (■). Curves were fitted with 4-PL regression analysis.



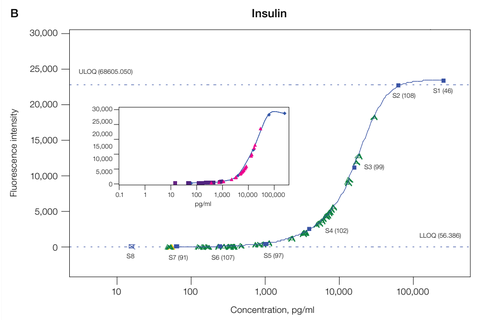


Fig. 5. Normal and diabetic mouse ghrelin (A) and insulin (B) levels fall within working assay range. Assay working range (80–120% standard recovery, 10% intra-assay CV) spans the measured concentration ranges of a typical set of unknown samples including fasting and fed animal samples. Results for other assays were similar. ■, standards; △, unknowns; insets: ■, normal samples; ▲, diabetes samples.

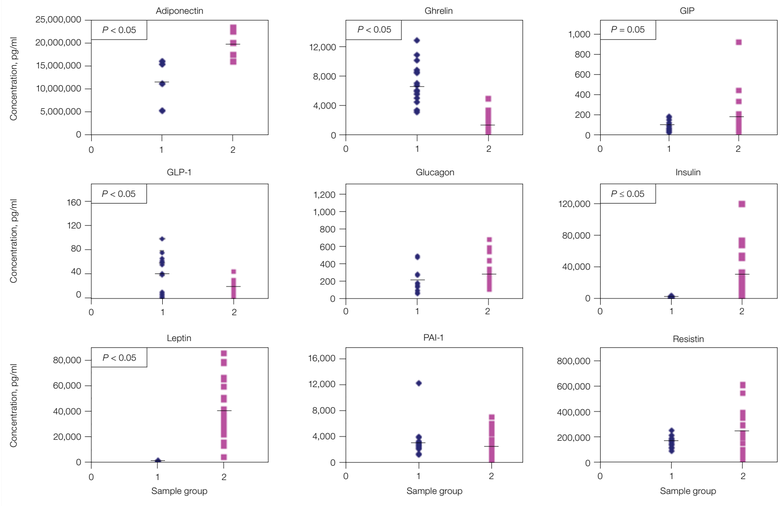


Fig. 6. Dot plot analysis of diabetic (n = 23) and normal (n = 16) mouse serum and plasma samples. One representative assay consisted of 39 mouse samples. The mean biomarker level for each group is marked with a black line. A Student's *t*-test established statistical significance between normal (◆) and type II diabetic (■) groups ($P \leq 0.05$) for established biomarkers.

Conclusions

- Bio-Rad Laboratories has developed a novel 8-plex assay, 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 required for preclinical research applications
- The Bio-Plex Pro mouse diabetes assays were developed on magnetic beads designed for automation and increased throughput. The assays are compatible with the automatic Bio-Plex Pro wash station for better precision and ease of workflow
- These mouse diabetes assays can be multiplexed with Bio-Plex Pro mouse cytokine and growth factor assays (i.e. 23-plex and 9-plex panels) for custom assay blends of varying plex levels
- The development and verification of these assays represent significant progress in improving multiplex assay quality and versatility required for diabetes research

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