Development of a Magnetic Bead-based Multiplex Immunoassay for Metabolic Biomarkers in Normal and Diabetic Mouse Serum and Plasma Samples

J. Eldering, A. Dulat, R. Zimmerman, V. Gupta, Bio-Rad Laboratories, Life Science, 2000 Alfred Nobel Dr., Hercules, CA 94547 USA

Life Science Group
2000 Alfred Nobel Drive
Hercules, CA 94547 USA

Abstract

Obesity and diabetes are on the rise and constitute a series of complex metabolic disorders with complex interactions among multiple proteins and environmental factors. Murine diabetes and obesity models exist to study these disorders through the measurement of circulating metabolic biomarkers. A particular challenge posed by these animal models is the limited amount of sample. To address this issue we have developed a novel multiplex immunoassay that can simultaneously measure eight of the most commonly measured metabolic biomarkers (insulin, leptin, PAI-1, resistin, ghrelin, GIP, GLP-1 and glucagon), as well as a single immunoassay for adiponectin, which requires a higher sample dilution than the other targets. The method uses the Bio-Plex® suspension array system, which is comprised of a flow-based 96-well fluorescent microplate assay reader integrated with specialized software. The Bio-Plex system uses an array of dye-coded microspheres to enable detection of multiple distinct analytes in as little as 12.5 µl of serum/plasma sample. The wash steps were automated using a Bio-Plex Pro™ wash station. The procedure for one plate was completed within 3 hours. The performance of the mouse metabolic biomarker assays were verified in three independent runs and found to be highly sensitive (LOD <0.5 to 179 pg/ml, depending on target), specific (<5% cross-reactivity), accurate (80–120% standard recovery), and precise (intra-assay <10% CV; inter-assay <15%). Sample linearity was good in both serum and plasma (R^2 = 0.87–1.0, depending on target) within assay range. Preliminary studies included simultaneous detection of eight circulating diabetes markers, and adiponectin, in normal and Type II diabetic mice. Results showed altered levels of key biomarkers in the disease state samples relative to the levels in the normal population. Furthermore, these metabolic biomarker immunoassays were found to be compatible for multiplexing with similar immunoassays for cytokines (i.e. TNFα, IL-6) that are implicated in inflammatory disorders and cancer.

Introduction

This mouse diabetes assay was developed to test multiple metabolic biomarkers simultaneously in three most common matrices, serum, plasma, and cell culture media. The objective of the assay development was to verify assay performance parameters and native sample recognition in normal and disease mouse models.

Method

Multiplex sandwich immunoassays were developed on 6.5 µm magnetic microspheres using the Bio-Plex suspension array system. This system integrates a series of color-coded magnetic beads each of which is coupled to a unique antibody specific for a distinct biochemical marker. The assays were performed in flat-bottomed 96-well plates using an automated magnetic wash station for wash steps. Multiplexed standard antigen, samples diluted 1 to 4 in sample diluent, and blank standard diluent were tested in triplicate wells. The procedure was conducted as described in Figure 1. The standard curves and samples were analyzed by 5-PL and 4-PL regression analysis using Bio-Plex Manager™ software version 6.0. The assays and analysis were completed within 3 hours. Assay performance parameters for Bio-Plex Pro mouse diabetes and adiponectin assays were verified in three independent assays. Samples tested were serum and plasma (K_2EDTA) collected from fed and fasted, normal (C57BL/6) and diabetic (DB/DB) mice.

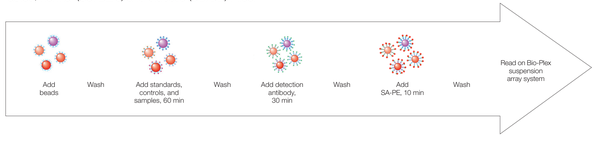

Fig. 1. Multiplex immunoassay workflow. The multiplex bead-based suspension sandwich immunoassay is conducted and read within 3 hours using a flow-based 96-well fluorescent microplate assay reader integrated with software. SA-PE, Streptavidin-Phycoerythrin.

Results

Standard Response Curves

Typical standard response curves of all nine analytes are shown in Figure 2. The standard analyte signal increases in proportion to increasing standard analyte concentrations over a 4 to 5 log range.

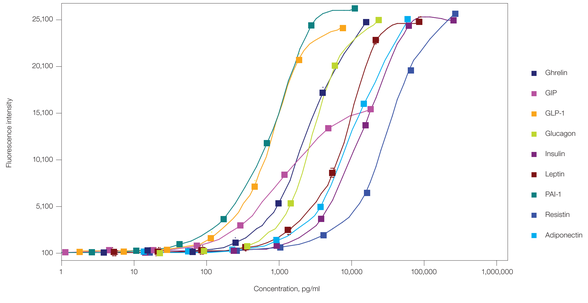

Fig. 2. Standard curves for Bio-Plex Pro mouse diabetes assay.

Specificity

The assays are highly specific with ≤1% cross reactivity among targets. 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.

Table 1. Cross-reactivity (%) among the targets in the Bio-Plex mouse diabetes panel.

Target	Adiponectin	Ghrelin	GIP	GLP-1	Glucagon	Insulin	Leptin	PAI-1	Resistin
Adiponectin	91	–0.1	–0.3	–0.4	–0.2	–0.4	–0.2	–0.1	–0.2
Ghrelin	0.0	94	–0.2	–0.3	–0.2	–0.1	–0.1	0.0	–0.4
GIP	–0.1	–0.1	98	1.4	–0.2	0.2	0.0	0.0	–0.2
GLP-1	0.0	–0.1	–0.3	96	–0.3	–0.1	0.1	0.0	–0.2
Glucagon	–0.1	–0.1	–0.4	–0.7	101	–0.1	–0.1	0.0	–0.2
Insulin	0.0	–0.1	–0.4	–0.9	–0.3	87	–0.1	0.0	–0.3
Leptin	0.0	–0.1	–0.3	–1.2	–0.4	–0.2	96	0.0	–0.4
PAI-1	0.1	0.0	–0.1	–0.7	–0.3	0.0	0.2	96	0.1
Resistin	0.0	0.0	0.0	0.0	0.0	–0.7	0.0	0.0	97

Limits of Quantitation, Sensitivity, and Precision

Overall assay performance was highly sensitive, accurate, and precise. The lower limit of quantitation (LLOQ), and upper limit of quantitation (ULOQ) values define the working assay range in which the assay is highly accurate (80–120% recovery) and precise (≤10% intra-assay CV). The lower limit of detection (LOD) is defined as the concentration obtained at the MFI that is 2-fold standard deviations above the background. 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 %CV was measured from the coefficient of variation of observed concentrations of eight standard points for three independent assays. The mean inter-assay %CV was obtained from standard points within assay range (80–120% recovery).

Table 2. Assay working range, sensitivity, and precision.

Targets	LLOQ	ULOQ	LOD	Intra-assay %CV	Inter-assay %CV
Adiponectin*	38.0	62,043	8.4	4	3
8-Plex Assays					
Ghrelin	3.1	7,296	0.8	5	4
GIP	13.4	14,999	2.3	4	10
GLP-1	3.4	1,969	0.8	6	11
Glucagon	24.0	3,067	7.0	6	6
Insulin	93.4	47,815	22.0	6	4
Leptin	17.1	69,900	6.2	4	3
PAI-1	0.7	2,922	0.5	5	2
Resistin	125.9	257,870	32.0	4	4

The LLOQ, ULOQ, LOD, and inter-assay precision %CV are the mean data determined from three independent multiplex assays in a serum-based matrix.
* Due to the different dilution scheme, adiponectin was assayed as a single assay.

Linearity

Linearity of dilution ensures that analytes present in concentrations within the assay range can be diluted and measured accurately for relative quantitation. The correlation coefficient (R^2) was determined by linear regression analysis of analytes measured in 3-fold serial dilutions of standard-spiked samples within assay range. Linearity (R^2 >0.99) was demonstrated in 3 matrices (mouse serum, plasma, and cell culture media) for all nine targets (Table 3).

Table 3. 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

Parallelism

Parallelism is necessary to reveal similar 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 3).

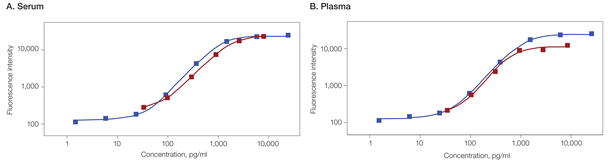

Fig. 3. Parallelism. Examples of parallelism for glucagon spiked in mouse serum (**A**) and plasma (**B**) between spiked samples (■) and standard curves (■). Curves were fitted with 4-PL regression analysis.

Sample Measurements within Standard Curve Range

Results from a representative 96-well plate assay shows analyte values from the majority of samples (n = 39; normal and diabetic mouse serum and plasma) were detected within the accurate range of the standard curve (80–120% standard recovery; ≤10% intra-assay CV; ≤15% inter-assay CV).

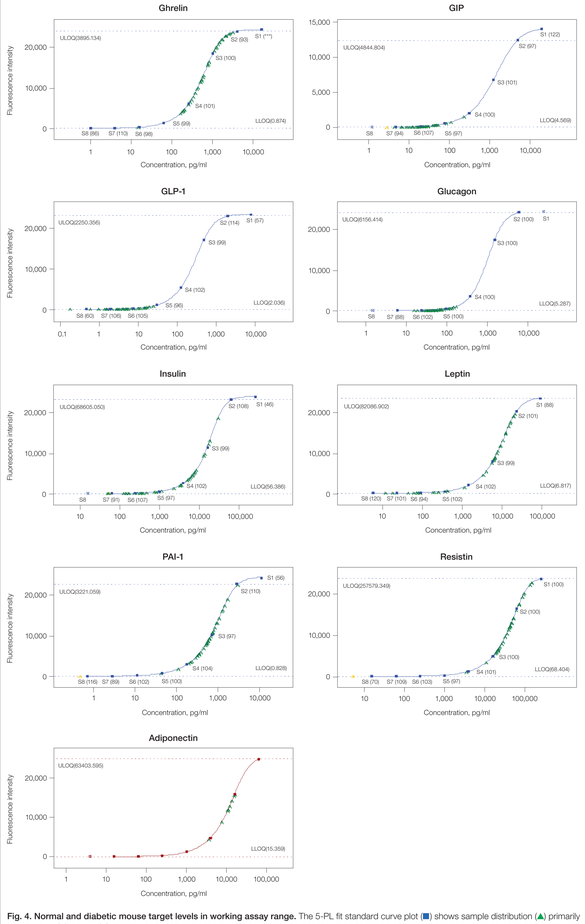

Fig. 4. Normal and diabetic mouse target levels in working assay range. The 5-PL fit standard curve plot (■) shows sample distribution (▲) primarily within the ULOQ and LLOQ (dotted lines). The % standard recovery (observed concentration/expected concentration x100) is indicated in parenthesis for each standard point.

Normal vs. Diabetic Mouse Plasma Sample Analysis: Fed and Fasted Conditions

Sample measurements of fed and fasted, normal and diabetic mouse groups (n = 10 each group) was determined in a representative 8-plex assay. Student's t-test (JMP ver 8 software) *P* value indicates anticipated statistical significances between normal and diabetic groups (P <0.05) for established biomarkers (i.e. ghrelin, GLP-1, insulin). Glucagon and insulin levels are significantly higher in fed diabetic mice but not in fasted diabetic mice, consistent with the disrupted metabolism in this model.

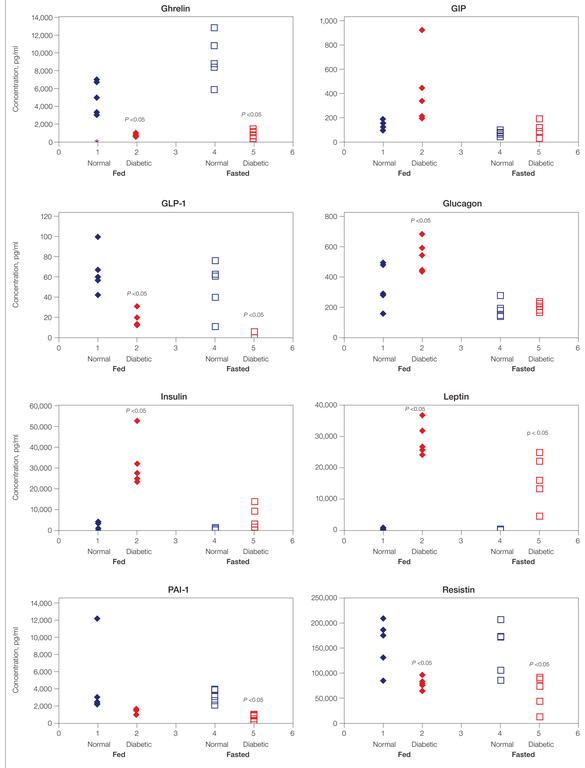

Fig. 5. Dot plot analysis of diabetic and normal mouse plasma samples collected under fed and fasting conditions.

Conclusion

- The magnetic bead-based Bio-Plex Pro mouse diabetes 8-plex assays allow simultaneous measurement of multiple diabetic and metabolic biomarkers in a single sample of serum, plasma or cell culture media matrices
- The assays have been optimized for high performance (specificity, sensitivity, accuracy, and precision) in conjunction with sample linearity, parallelism, and a broad assay range required for preclinical research applications
- The implementation of magnetic bead-based assays allows for automation of assay wash steps using available magnetic washers and the complete robotic automation of assay processing
- The development and verification of these assays represent significant progress in improving multiplex assay quality required for diabetes research

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