Application Note

A new MILLIPLEX® MAP multiplex panel enhances simultaneous quantitation of metabolic hormones in human serum and plasma

Obesity, diabetes, cardiovascular disease and associated conditions under the umbrella of Metabolic Syndrome are urgent health issues. Hormones produced by various organs of the endocrine system, including adipocytes, the pancreas and the gastrointestinal tract, play an integrated role in regulating energy, food intake and whole body metabolism (Figure 1). With the incidence of type 2 diabetes on the rise even among children and teens, and with links being made between metabolic signaling and tumor progression, the study of hormones linked to metabolic syndrome is essential to both treatment and prevention.

Because multiple signals are required to maintain the system in a balanced state, no single biomarker is sufficient to define a system. On the other hand, measuring multiple analytes, one at a time, can be inefficient or impossible because of sample volume limitations. To overcome these challenges, we developed a multiplex immunoassay panel, based on Luminex xMAP® magnetic bead-based technology, that enabled the simultaneous quantitation of metabolism-relevant analytes[1]. In this application note, we describe a new, improved metabolic hormone magnetic bead-based assay panel that enables the detection of 13 analytes, including:

- Pancreatic hormones: amylin (active or total), C-peptide, glucagon, insulin, and pancreatic polypeptide (PP)
- Gut hormones: active ghrelin, gastric inhibitory peptide (GIP), glucagon-like peptide 1 (GLP-1, active or total), and peptide YY (PYY)
- Adipokines: leptin, IL-6, monocyte chemotactic protein-1 (MCP-1), and tumor necrosis factor α (TNFα)

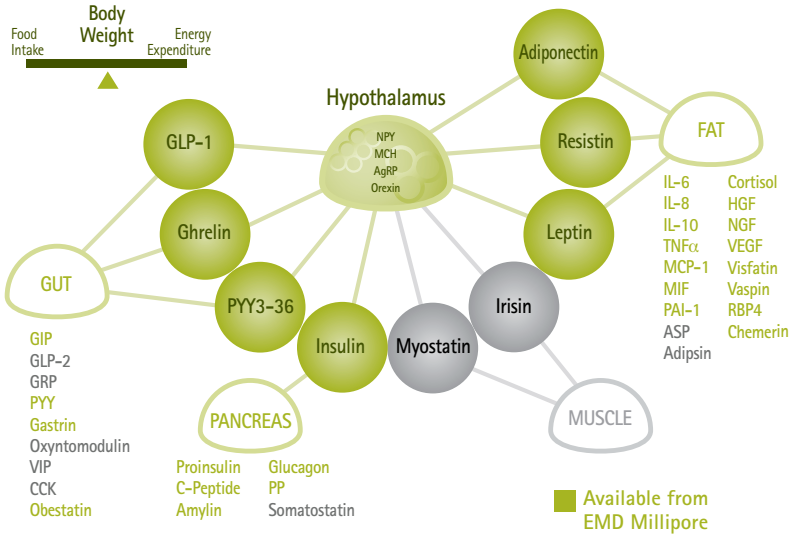

Figure 1.
Multiple signals secreted to the brain by various organs contribute to maintaining the balance between food intake and energy expenditure, to regulate body weight and metabolism.

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These immunoassays were developed using magnetic bead-based Luminex xMAP® technology, which consists of a capture antibody immobilized on magnetic beads and a biotinylated detection antibody. Magnetic bead-based assays provide several advantages over non-magnetic bead-based assays, including easier automation and high-throughput screening, more flexible plate and plate washer options and elimination of technical obstacles (i.e., clogging of wells) which may result from vacuum manifold/manual washing.
One key advantage of this metabolic hormone assay panel is the inclusion of an assay for total GLP-1 and a high-sensitivity assay for active GLP-1. In addition to evaluating the accuracy and precision of the entire panel, we compared the performance of the high sensitivity GLP-1 active MILLIPLEX® MAP assay to the results obtained using the established, high sensitivity GLP-1 active ELISA (Cat. No. EZGLPHS-35K).

Materials and Methods

Multiplex assays were conducted using the MILLIPLEX® MAP Human Metabolic Hormone Magnetic Bead Panel (Cat. No. HMHEMAG-34K), following the instructions in the included protocol.

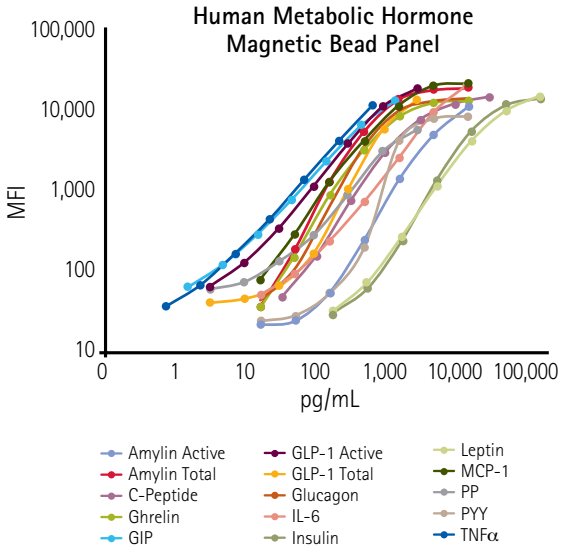
To determine the changes in metabolic hormone levels with respect to food intake, blood samples were collected from normal subjects (n=20) after overnight fasting or one hour after eating a standard breakfast. The protease inhibitors (DPPIV inhibitor, AEBSF, protease inhibitor cocktail) were immediately added in the collected blood samples. The serum and plasma samples were separated and measured with the MILLIPLEX® MAP Human Metabolic Hormone Magnetic Bead Panel according to the assay protocol.

The same samples were also evaluated using the High Sensitivity GLP-1 Active ELISA Kit, Chemiluminescent (Cat. No. EZGLPHS-35K) using the included protocol.

Results

Using standards for each analyte serially diluted in serum matrix, standard curves were prepared to determine the assay response (measured in Mean Fluorescence Intensity (MFI)) with respect to analyte concentration (Figure 2A). The standard curves established linearity of the assay over three to five orders of magnitude for all analytes (Figure 2B). No cross-reactivity was seen within the panel (data not shown). The assays showed high recoveries (94%-107%) and good precision (less than 10% intra-assay coefficients of variation (CV) and 15-20% inter-assay coefficients of variation (Figure 2B).

2A.



2B.

Analyte	Std Range (pg/mL)	Sensitivity (MinDC)	Recovery in serum matrix (%)	Intra-assay CV (%)	Inter-assay CV (%)
Amylin Active	14 - 10,000	16	97	<10	<20
Amylin Total	14 - 10,000	13	104	<10	<20
C-Peptide	27 – 20,000	9.5	99	<10	<15
Ghrelin	14 -10,000	14	105	<10	<15
GIP	1.4 – 1,000	0.6	103	<10	<15
GLP-1 Active	2.7 – 2,000	1.2	99	<10	<15
GLP-1 Total	2.7 – 2,000	2.5	103	<10	<20
Glucagon	14-10,000	13	101	<10	<15
IL-6	2.7 – 2,000	11	94	<10	<15
Insulin	137 – 100,000	87	97	<10	<15
Leptin	137 – 100,000	41	102	<10	<15
MCP-1	14 – 10,000	14	106	<10	<15
PP	2.7 – 2,000	2.0	104	<10	<15
PYY	14 – 10,000	90	107	<10	<15
TNFα	0.7 - 500	0.3	97	<10	<15

Figure 2.

Standard curves (2A) and validation data (2B) calculated using analytes serially diluted in matrix and analyzed using the MILLIPLEX® MAP Human Metabolic Hormone Magnetic Bead Panel (Cat. No. HMHEMAG-34K).

Next, we evaluated the performance of the multiplex panel in biological samples. As expected, the levels of multiple metabolic hormones changed in response to ingestion of food (increases in serum C-Peptide, GIP, active GLP-1 and Insulin shown in Figure 3A. The comparison of matched human serum and plasma samples showed that the sample concentrations in serum and plasma are very comparable for majority of the analytes (representative analytes shown in Figure 3B).

3A.

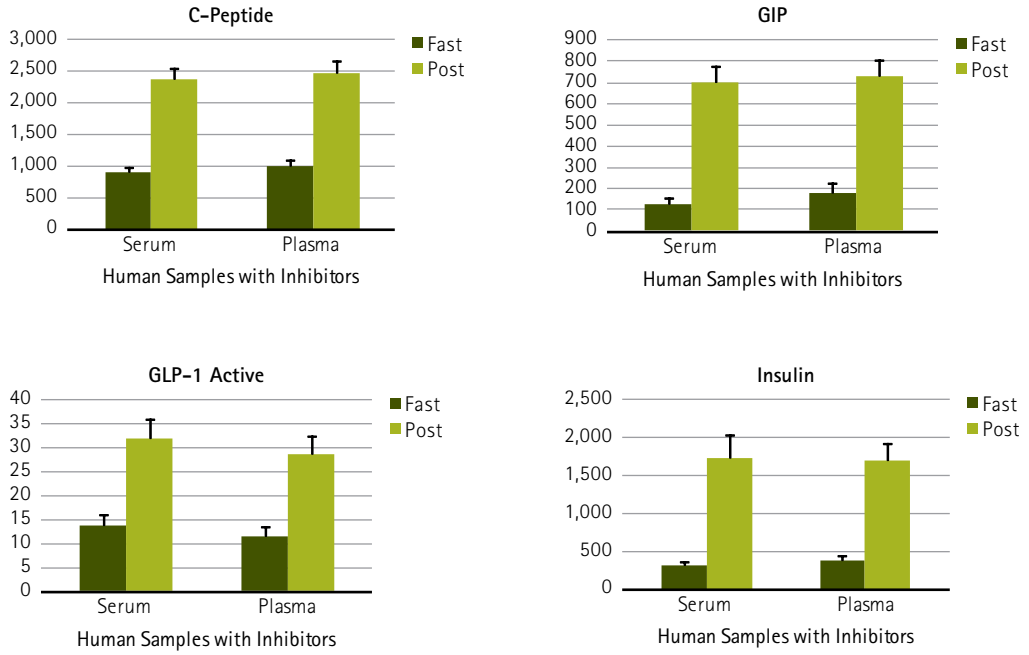
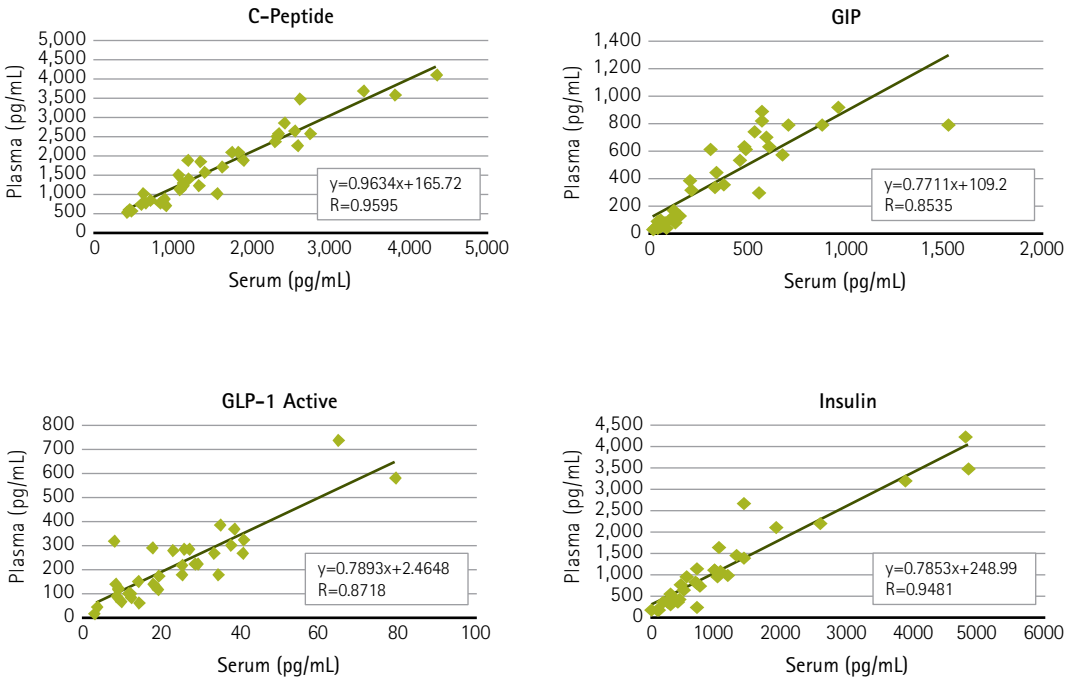


Figure 3.

Multiplexed analysis of metabolic hormones in human serum (with protease inhibitors) and plasma from fasting or post-prandial subjects. Expected increases were seen in serum C-Peptide, GIP, active GLP-1 and Insulin (3A). Comparison of serum analyte levels to plasma analyte levels showed good correlation as seen by the slope and R values in the correlation curves (3B).

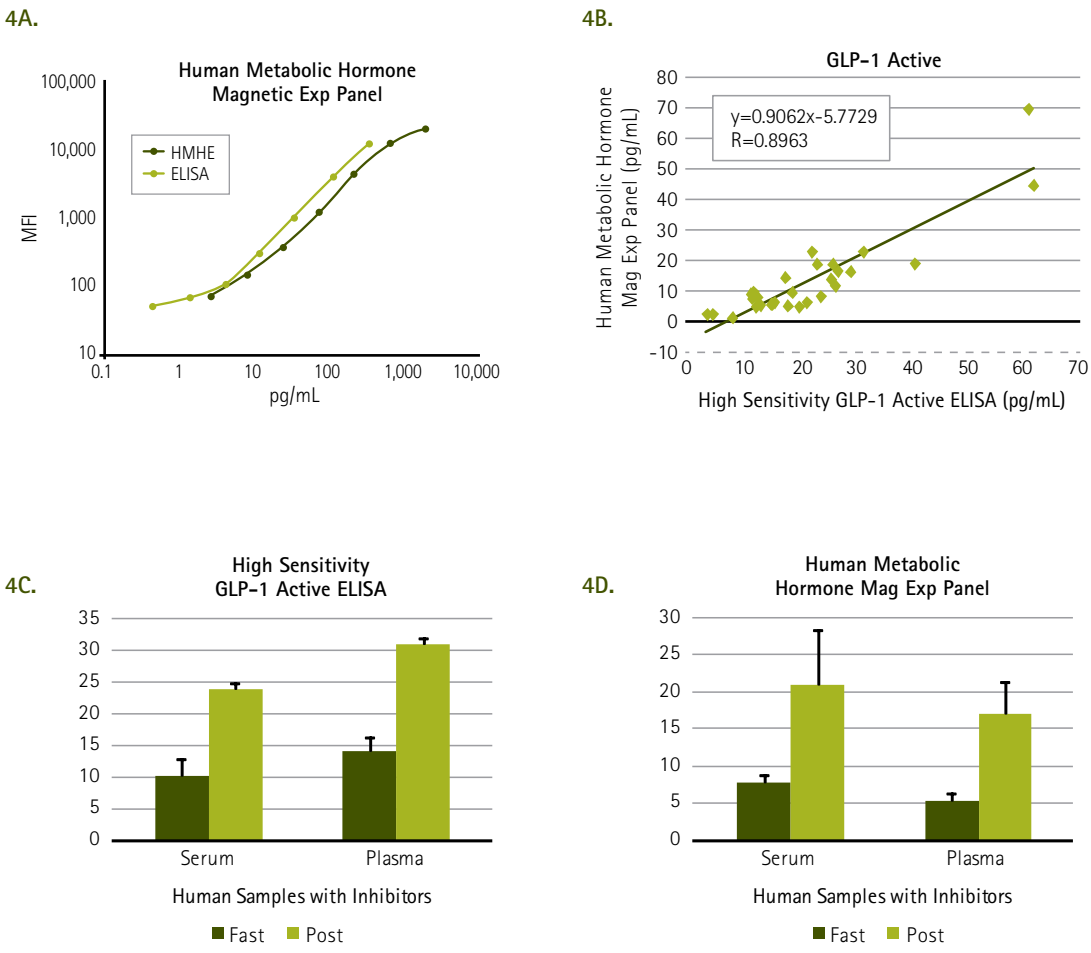
3B.



We compared the quantitation of active GLP-1 using the MILLIPLEX® MAP Human Metabolic Hormone Magnetic Bead Panel with GLP-1 quantitation using the established method, EMD Millipore's high sensitivity active GLP-1 ELISA kit (Figure 4). Standard curves created using serially diluted standards of purified GLP-1 showed extremely similar, nearly overlapping assay response and linear range of the assays (Figure 4A). Comparing measured levels of GLP-1 in biological samples using the MILLIPLEX® MAP assay with results obtained from the ELISA kit (Figure 4B) showed good correlation between the two methods, with slope and R value approaching unity. Finally, both methods showed the expected increase in GLP-1 with respect to food intake (Figure 4C and 4D).

Figure 4.

Correlation between MILLIPLEX® MAP GLP-1 (active) assay results and high sensitivity GLP-1 (active) ELISA results for GLP-1 standard (4A), serum samples (4B) and comparison of fasting to postprandial subjects (4C and 4D).



Finally, we compared metabolic hormone levels measured using three different methods: the MILLIPLEX® MAP magnetic bead assay panel, an assay panel from Supplier A that was not based on Luminex® technology, and a Luminex® technology-based assay panel from Supplier B.

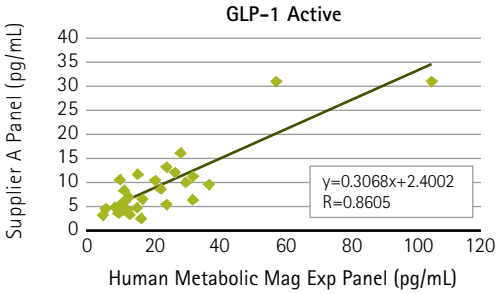
Figure 5 shows the comparison between the MILLIPLEX® MAP magnetic bead assay panel and the non-Luminex® assay panel from Supplier A. Note that Supplier A's assay required 40 µL of sample, while the MILLIPLEX® MAP assay required only 25 µL. Comparing the assay characteristics (Figure 5A) showed that assay sensitivity and dynamic range were comparable for representative analytes. Comparison of the data obtained (representative analytes shown in Figure 5B) also showed that the assay platforms, in general, gave well-correlated analyte quantitation, with the exception of glucagon and leptin. For these analytes, data from each assay platform was compared with ELISA quantitation results, and it was found that the MILLIPLEX® MAP assay results matched the ELISA results more closely than did Supplier A's assay (data not shown)[13]. We assessed the biological relevance of the new MILLIPLEX® MAP GLP-1 (active) assay as well as Supplier A's GLP-1 (active) assay by comparing calculated analyte levels with ELISA data (Figure 5C and 5D) and found very good correlation (R=0.89 and R=0.91, respectively).

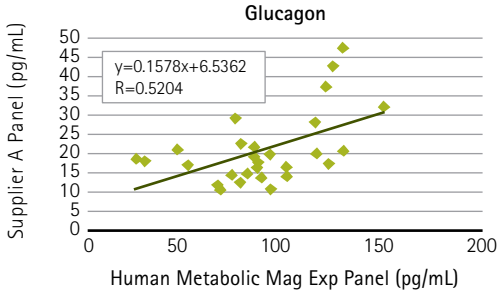
5A.

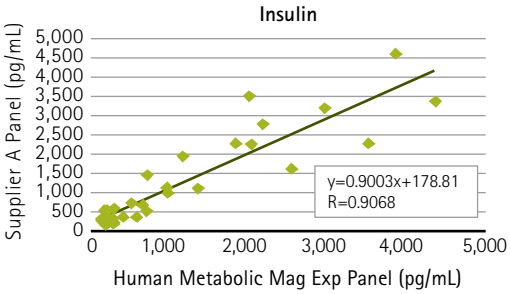
EMD Millipore Supplier A

Analyte	Std Range (pg/mL)	Sensitivity Min DC* (pg/mL)	Std Relative Potency	Sample Detectability (%, N=32)	Sample Volume (µL)	Assay Incubation
GLP-1 Active	2.7 – 2,000	0.7	1	88	25	Overnight 20 hrs
GLP-1 Active	2.4 – 10,000	2.1 1 (LLOD)	2.5	100	40	5 hrs
Glucagon	14 – 10,000	11	1	100	25	Overnight 20 hrs
Glucagon	2.4 -10,000	3.4 20 (LLOD)	0.5	88	40	5 hrs
Insulin	137 – 100,000	30	1	100	25	Overnight 20 hrs
Insulin	12 – 50,000	9.8 9 (LLOD)	1.2	100	40	5 hrs
Leptin	137 – 100,000	2	1	100	25	Overnight 20 hrs
Leptin	24 – 100,000	25 22 (LLOD)	0.7	100	40	5 hrs

5B.







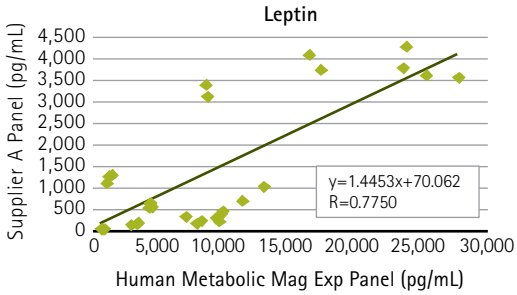
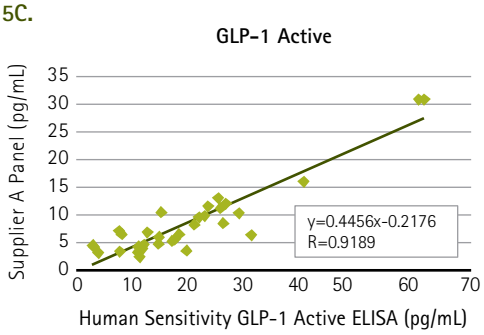


Figure 5.

Comparison between MILLIPLEX® MAP (MM) multiplex assay and a different assay from Supplier A not based on Luminex® technology. We compared assay characteristics (5A) and calculated concentrations (5B) for representative analytes. Quantitation of active GLP-1 using both assays was compared with ELISA data (5C and 5D).

5C.



5D.

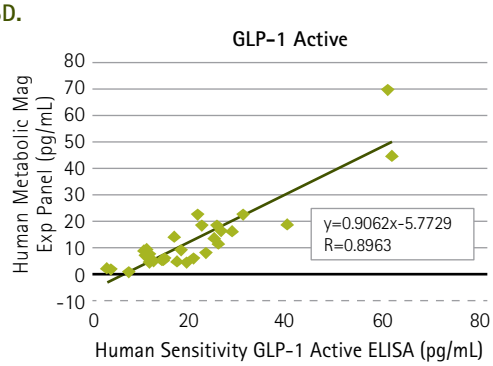


Figure 6 shows the comparison between the MILLIPLEX® MAP magnetic bead assay panel and another Luminex® technology-based assay from Supplier B. On average, the two assay panels provided similar sensitivity and range (Figure 6A). The MILLIPLEX® MAP assay protocol was slightly simpler and introduced less potential pipetting error, compared to Supplier B's assay, which required dilution of the sample. Figure 6B showed well-correlated analyte quantitation using the two assays for C-peptide, GIP and insulin, but very poor correlation for active GLP-1, glucagon, and leptin. We believe our active GLP-1, glucagon and leptin sample values were more accurate, because these assay data were better correlated with ELISA results[13]. Moreover, active GLP-1 and glucagon levels measured using MILLIPLEX® MAP assays were consistent with reported sample values in publications[2-12], while the sample values generated using Supplier B's panel were much higher than published data.

6A.

EMD Millipore Supplier B

Analyte	Std Range (pg/mL)	Sensitivity Min DC* (pg/mL)	Std Relative Potency	Sample Detectability (%, N=32)	Sample Volume (µL)	Assay Incubation
C-Peptide	27 – 20,000	13	1	100	25	Overnight 20 hrs
C-Peptide	1 – 16,010	2.5	0.9	100	50 (1:4)	2 hrs
Ghrelin	14 – 10,000	11	1	31	25	Overnight 20 hrs
Ghrelin	2.3 – 37,841	6.7	0.3	100*	50 (1:4)	2 hrs
GIP	1.4 – 1,000	0.1	1	100	25	Overnight 20 hrs
GIP	1.3 – 21,234	0.4	0.5	100	50 (1:4)	2 hrs
GLP-1 Act	2.7 – 2,000	0.7	1	88	25	Overnight 20 hrs
GLP-1 Act	1.7 – 28,531	3.7	0.5	100*	50 (1:4)	2 hrs
Glucagon	14 – 10,000	11	1	100	25	Overnight 20 hrs
Glucagon	2.5 – 41,012	2	0.33	100*	50 (1:4)	2 hrs
Insulin	137 – 100,000	30	1	100	25	Overnight 20 hrs
Insulin	1.8 -29,962	3.2	0.83	100	50 (1:4)	2 hrs
Leptin	137 – 100,000	2	1	100	25	Overnight 20 hrs
Leptin	18 – 297,959	8.7	0.8	100	50 (1:4)	2 hrs

6B.

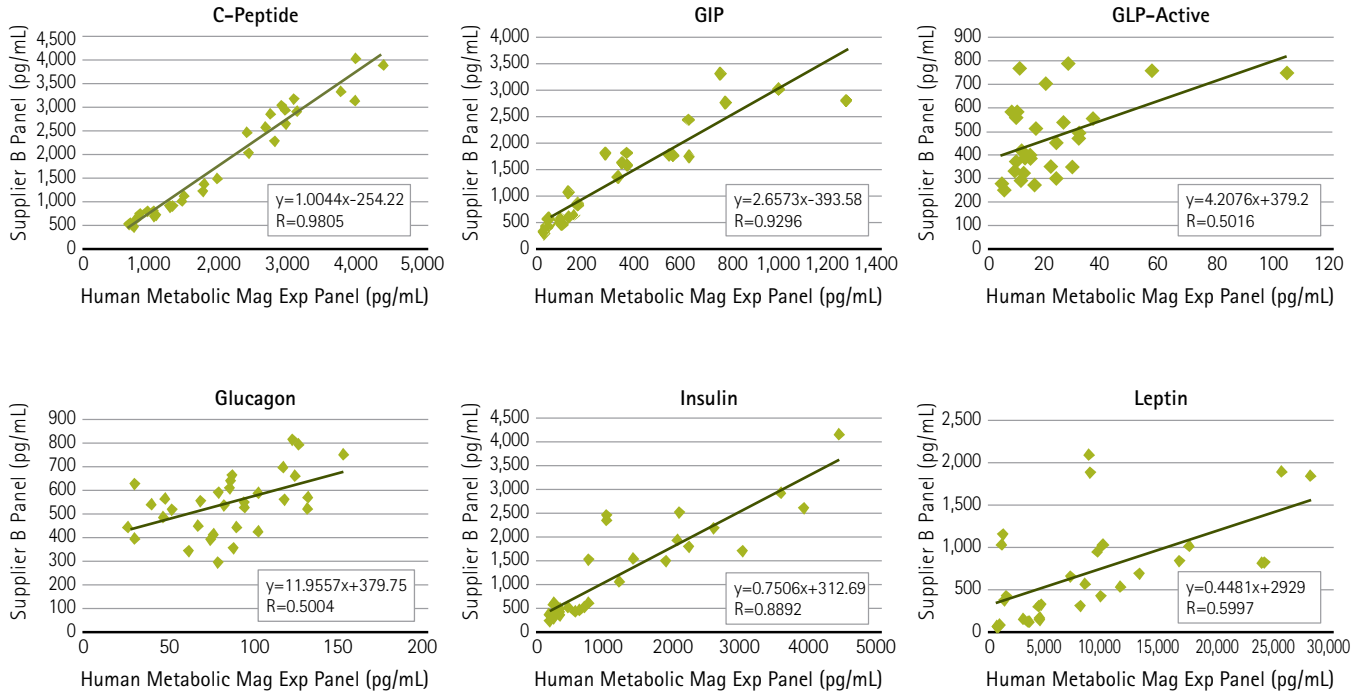


Figure 6.

Comparison between MILLIPLEX® MAP (MM) multiplex assay and a different Luminex®-based assay from Supplier B. We compared assay characteristics (6A) and calculated concentrations (6B) for representative analytes.

Conclusion

The new MILLIPLEX® MAP Human Metabolic Hormone Panel is a second-generation multiplex panel for the simultaneous quantitation of crucial metabolism analytes. Compared to the previous MILLIPLEX® MAP human metabolic panel (Cat. No. HMHMAG-34K), the new panel features more sensitive detection of active GLP-1 by using a new antibody pair. Addition of a Total GLP-1 assay enables the user to select either active GLP-1 or total GLP-1 for this panel. The sample data of these two analytes are highly correlated to ELISAs.

Our analytical and biological validation data have demonstrated that this multiplex panel is sensitive, accurate, and reproducible. The sample values generated with this new panel are consistent with the first generation panel (Cat. No. HMHMAG-34K) and Human Gut Panel (Cat. No. HGT-68K).

Comparing this new Human Metabolic Hormone Panel with other commercially available multiplex assays revealed that this kit is more user-friendly and robust, and the assay performance is similar or better than kits from other suppliers. This new panel has the potential to be a very useful tool for basic research and clinical studies in the areas of metabolic disease and other related pathophysiologies.

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