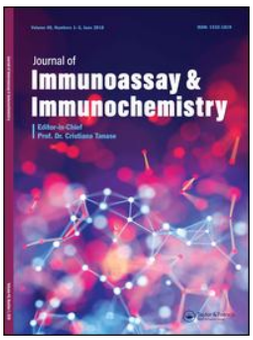

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ELISA versus LUMINEX assay for measuring mouse metabolic hormones and cytokines: sharing the lessons I have learned

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ABSTRACT

Enzyme-linked immunosorbent assay (ELISA) has long been the standard for quantitative analysis of metabolic hormones and cytokines. LUMINEX multiplex bead array assays were developed as cost- and time-effective alternatives to ELISA, but they are the only cost- and time-effective if they provide informative data. Here, I show that using half-volume of reagents for an adiponectin single-plex LUMINEX assay and a 6-plex LUMINEX xMAP mouse metabolic bead assay, produces reliable data and increases assay cost-effectiveness. I provide direct comparisons between LUMINEX assay and ELISA for quantitation of mouse leptin and insulin, and evaluate glucagon, GLP-1, IL-6, and TNFα data obtained using the 6-plex LUMINEX assay for a high-fat diet-induced obesity study. Good correlations between assays were obtained for fasting leptin and non-fasting insulin. However, the LUMINEX assay proved unsuitable for quantitating fasting insulin. ELISA proved suitable for quantitating fasting male, but not female, insulin. The LUMINEX assay gave lower values for leptin and higher values for insulin, compared with ELISA. The mouse metabolic LUMINEX assay proved unsuitable for quantitating glucagon, GLP-1, IL-6, and TNFα, due to undetectable levels in most fasting and non-fasting plasma. Overall, quantitative leptin levels were the only reliable data obtained from the mouse metabolic LUMINEX assay.

KEYWORDS

ELISA; LUMINEX; mouse metabolic hormones; insulin; leptin; adiponectin; IL-6; TNFα

Introduction

Accurately quantitating multiple plasma protein levels is informative in metabolic research and diagnostics. ELISA has long been the standard for quantitative analysis of metabolic hormones and cytokines. However, measuring multiple targets using ELISA requires a separate ELISA for each target, and therefore is time-consuming and expensive. Furthermore, the sample volume required increases with the number of targets to be quantitated using ELISAs (~50-100 µL per sample) and this becomes limiting for mouse plasma samples, which are often scarce. In contrast, LUMINEX multiplex bead array assays

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provide quantitative measurements of multiple targets simultaneously (~total of 25–50 μL for multiple samples) and are therefore considered cost- and time-effective.

Key differences between ELISA and LUMINEX assays are the use of different target capture systems and different reporter systems. LUMINEX assays capture targets onto spherical beads in suspension while ELISAs generally rely on flat surfaces in multi-well plates to capture targets. LUMINEX assays use fluorescence as a reporter system whereas ELISAs use enzyme amplification of a colorimetric substrate. The LUMINEX multi-analyte-profiling (xMAP) technology uses digital signal processing capable of classifying polystyrene beads dyed with distinct proportions of red and near-infrared fluorophores. These proportions define 'spectral addresses' for each bead population.

There are few peer-reviewed publications investigating whether the quantitative data obtained by LUMINEX xMAP multiplex bead array assays are identical to, or at least similar to, data obtained using ELISA. A limited number of direct comparisons between LUMINEX xMAP multiplex bead assays and ELISA for quantitation of cytokines have been reported.[1–4] However, I have not found any direct comparison studies for LUMINEX xMAP multiplex bead assays versus ELISA for metabolic or gut hormones. Overall for cytokine assays, good correlations, but poor concurrence of quantitative values, between LUMINEX multiplex bead array assays and corresponding ELISA values have been reported.[4]

LUMINEX assay kits are expensive and require expensive specialized equipment relative to ELISA kits. Therefore, direct comparisons between LUMINEX and ELISA kits would benefit researchers having to decide which assay is best to use to maximize the information obtained for mouse metabolic studies.

The metabolic syndrome includes obesity, diabetes, and co-morbidities such as heart disease and kidney disease, all of which are linked with inflammation. Biomarkers for metabolic syndrome include obesity and diabetes-related hormones, gut hormones, and cytokines. Therefore, a LUMINEX assay kit that simultaneously measures multiple metabolic hormones using small sample volumes has potential to be more informative for a metabolic study than using ELISAs.

Three aspects of these assays were evaluated here. First, I investigated whether LUMINEX assays could be used to reliably measure plasma samples if only half of the plasma and assay volumes stipulated in the manufacturer's protocol were used. Therefore, full and half-volume standards, QCs, and mouse plasma were compared for an adiponectin single-plex Luminex and also for the 6-plex LUMINEX xMAP mouse metabolic bead assay. The purpose was to make these assays more cost-effective, since ~80% more samples could be measured using a LUMINEX assay kit if half volumes were used. Second, I compared quantitation of leptin and insulin levels in the same

plasma samples in both ELISA and the 6-plex LUMINEX kits. The purpose was to determine whether, and how, quantitation of mouse insulin and leptin levels is dependent on the type of assay used. Third, I investigated whether publishable data could be obtained using the 6-plex LUMINEX xMAP mouse metabolic bead assay kit to quantitate glucagon, glucagon-like peptide-1 (GLP-1), interleukin-6 (IL-6) and tumor necrosis factorα (TNFα) in mouse plasma obtained from a previous study which characterized high-fat diet-induced obesity in a genetic model for mouse obesity.[5]

Finally, based on my experience with these assays and these data analyses, I highlight lessons that I learned, and my recommendations for researchers in the future who are faced with deciding between LUMINEX and ELISA kits to measure biomarkers for mouse metabolic syndrome.

Materials and methods

Animals

All samples used for data analysis were collected from a previous chronic high-fat diet-induced obesity mouse study.[5] All experimental procedures involving mice were approved by the University of Auckland Animal Ethics Committee and conformed to The Animal Welfare Act 1999. The previous study included male (M) and female (F) C57BL/6 J mice with a targeted mutation in the *Pomc* gene (*Pomc*$^{tm1/tm1}$ mice; mice are unable to synthesize desacetyl-α-melanocyte stimulating hormone [MSH] and α-MSH) and their wild-type (WT) littermates. There was a range of metabolic energy states for these mice; *Pomc*$^{tm1/tm1}$ mice were obese compared with lean C57BL/6 J WT littermate mice, different cohorts of mice were fed low-fat (LF; 10 kcal% fat) or high-fat (HF; 45 kcal% fat) diet for up 23 weeks post-weaning, and some male *Pomc*$^{tm1/tm1}$ mice were treated centrally with continuous desacetyl-α-MSH or α-MSH (using osmotic mini pumps) intracerebroventrical (*icv*) administered over 14 days to reverse obesity.[5] All mice were fasted overnight before being euthanized, except the male *Pomc*$^{tm1/tm1}$ mice that were treated *icv* to reverse obesity. Therefore, wide ranges of values for metabolic and gut hormones and cytokines were expected for analysis. ELISA was used to quantitate the plasma for leptin and insulin and as expected, a wide range of values were found. These data were previously published for the characterization of the chronic high-fat diet-induced obesity study.[5] These same data were used here to compare leptin and insulin measured using ELISA with measurements made using 6-plex LUMINEX xMAP mouse metabolic bead assay.

Blood collection

Blood was collected either by cardiac puncture from anesthetized mice, or by using Goldenrod animal lancets (Medipoint International, Inc., NY) to collect

blood from the submandibular vein of conscious mice aged approximately 19 weeks.[5] Fasting or non-fasting blood samples were collected from different cohorts of mice that had been fed either LF or HF diets since weaning.[5] Blood was added to ice-cold EDTA-coated microtainer tubes and centrifuged at 4000 g for 10 min at 4°C. Plasma was then transferred to ice-cold low-bind Eppendorf® tubes (Sigma-Aldrich, New Zealand Ltd) containing DPP-IV inhibitor (10 µL; MPDPP4, Merck Millipore, Auckland, New Zealand), pefabloc SC (10 µL/0.01 g; 114298001, Roche Diagnostics Ltd.), and protease inhibitor cocktail (10 µL; P8340, Sigma-Aldrich). Multiple 20 µL, 30 µL, or 40 µL aliquots of each plasma were frozen and stored at −20 C. The majority of samples were thawed only once or twice before assay.

Study 1: Can LUMINEX (MILLIPLEX) assays be used to reliably quantitate plasma samples if only half of the plasma and assay volumes stipulated in the manufacturer's protocol are used?

The aim was to make LUMINEX assays more cost-effective since ~80% more samples can be measured using a LUMINEX assay kit if half volumes are used. Adiponectin was measured using MILLIPLEX® MAP kit with mouse adiponectin magnetic bead single plex (#MADPNMAG-70 K-01). A 6-plex LUMINEX xMAP mouse metabolic bead assay (# MMHMAG-44 K, Millipore Corporation, MA) was used to quantitate plasma leptin, insulin, glucagon, GLP-1(active), interleukin-6 IL-6, and TNFα. To determine whether these assays could be used with half of the volumes of reagents required by the manufacturer, standards, and two quality controls (QCs) provided with each kit were assayed following the manufacturer's protocol alongside standards and QCs assayed using half-volumes of all reagents, except drive fluid. Drive fluid (100 µL per well) remained unchanged from the manufacturer's protocol. The assays were analyzed using a MAGPIX with xPONENT software by LUMINEX corporation. The linearity of the standard curves and intra- and inter-assay coefficient of variation (CV) were compared for full- versus half-volume assays. The standard ranges and sensitivities for detection of each analyte measured using full-volume samples (10 µL/well) according to the manufacturer's protocol are shown in Table 1.

Study 2: Is quantitation of mouse leptin or insulin,dependent on whether the sample is measured using LUMINEX or ELISA?

The aim was to compare quantitation of leptin and insulin levels in the same plasma samples (different aliquots) measured using ELISA and MILLIPLEX® MAP kit with 6-plex mouse metabolic magnetic bead panel. The metabolic phenotype of WT *Pomc*$^{tm1/tm1}$ mice characterized for chronic high-fat diet-induced obesity was previously published.[5] Overnight fasting plasma taken

Table 1. Comparison between ELISA and LUMINEX assays for ranges of standards and manufacturer reported sensitivity. The manufacturer's standard ranges and assay sensitivities are shown for leptin and insulin ELISA, single-plex MILLIPLEX® MAP adiponectin kit, and MILLIPLEX® MAP kit with 6-plex mouse metabolic magnetic bead panel for leptin, insulin, glucagon, GLP-1, IL-6, and TNFα. The ranges of quantitative levels for each hormone or cytokine reported in publications where ELISA or LUMINEX assay kits were used are shown for fasted and fed mouse plasma. These reported ranges show that many published studies are reporting values which are outside of the standard range for the LUMINEX assay used here.

ELISA	Standard Range	Sensitivity (Assay with 10 µL plasma)	Published data for fasted mouse sample	Published data for fed mouse sample
Leptin	0.02–30.00 ng/mL (~ 0.125–188 pM)	0.05 ng/mL	1–19 ng/mL[6]	
Insulin	0.10–10.00 ng/mL (~ 17.5–175 pM)	0.10 ng/mL	0.1–0.8 ng/mL[6]	
LUMINEX	Standard Range	Sensitivity (Assay with 10 µL plasma		
Adiponectin	12.2–12,500 pg/mL	3 ± 5 pg/mL	25–35 µg/mL[7]	
Leptin	69–50,000 pg/mL (0.069–50 ng/mL)	19 ± 40 pg/mL (0.019 ng/mL)	600–750 pg/mL[7]; ~25 pg/mL[8]	~ 790 pg/mL[9]
Insulin	69–50,000 pg/mL (0.069–50 ng/mL)	14 ± 30 pg/mL (0.014 ng/mL)	~ 600 pg/mL (male)[8]	1.3–2.0 ng/mL[10]
Glucagon	14–10,000 pg/mL	7 ± 15 pg/mL	< 80 pg/mL[11]	25–100 pg/mL[10]
GLP-1	41–30,000 pg/mL	23 ± 41 pg/mL	< 10 pg/mL[11]	Not detected[12]; ~50 pg/mL[9]
IL-6	27–20,000 pg/mL	8 ± 15 pg/mL	50–80 pg/mL[7]	5–10 pg/mL[12]; Not detected[9]
TNFα	27–20,000 pg/mL	8 ± 16 pg/mL	5–7 pg/mL[7]	19–31 pg/mL[12]; ~15 pg/mL[9]

from male and female WT and *Pomc*$^{tm1/tm1}$ mice fed LF or HF diets for 23 weeks post-weaning in this previous study were assayed with ELISA for leptin and insulin. These data were compared with LUMINEX assay data generated in this study on the same plasma samples.

Also, in the previous study, male *Pomc*$^{tm1/tm1}$ mice fed LF or HF diets for 21 weeks post-weaning had vehicle, 5 µg α-MSH/25 g body weight or 5 µg desacetyl-α-MSH/25 g body weight continuously administered *icv* over 14 days before mice were euthanized and non-fasting blood collected.[5] These plasma samples previously measured for leptin and insulin using ELISA, were compared with LUMINEX assay data generated in this study on the same plasma samples.

Plasma insulin and leptin were measured by ELISA kits (# EZRMI-13 K and # EZML-82KEMD, Millipore Corporation, MA) using 10 µL/standard or sample and by MILLIPLEX® MAP kit with 6-plex mouse metabolic magnetic bead panel using 5 µL/standard or sample. The standard ranges and sensitivities for detection of each analyte measured according to the manufacturer are shown in Table 1. Measurements on duplicates of standards and samples were performed and if the CV% for a duplicate sample was >5%, the sample was measured in a repeat assay.

Statistics

GraphPad Prism v7 software (GraphPad Software, San Diego, CA, USA) was used to perform statistical analyses. Pearson's correlation analysis was used to examine the relationships between ELISA and LUMINEX data for leptin and insulin. Linear regression analysis and Bland-Altman analysis were used to calibrate ELISA against LUMINEX. For statistical tests, a probability level of $p < .05$ was regarded as significant. All values are expressed as mean ± SEM.

Study 3: Can reliable quantitative levels for plasma glucagon, GLP, IL-6 and TNFα be obtained using the MILLIPLEX® MAP kit with 6-plex mouse metabolic magnetic bead panel, that would contribute to characterization of a mouse metabolic phenotype?

The aim was to determine whether the MILLIPLEX® MAP kit with 6-plex mouse metabolic magnetic bead panel used to quantitate plasma leptin and insulin for Study 2, could also provide meaningful quantitation for glucagon, GLP-1, IL-6, and TNFα toward characterization of a phenotype for chronic HF diet-induced mouse obesity. Overnight fasting plasma taken from male and female WT and *Pomc*$^{tm1/tm1}$ mice fed LF or HF diets for 23 weeks post-weaning were assayed in study. The metabolic phenotype of these mice was previously published.[5] The MILLIPLEX® MAP kit with 6-plex mouse metabolic magnetic bead panel was used to quantitate leptin, insulin, glucagon, GLP-1, IL-6, and TNFα in these samples. In Study 3, these fasting plasma glucagon, GLP-1, IL-6, and TNFα levels were analyzed for obese-genotype and diet effects.

In the previous published study, male *Pomc*$^{tm1/tm1}$ mice fed LF or HF diets for 21 weeks from weaning had vehicle, 5 µg α-MSH/25 g body weight or 5 µg desacetyl-α-MSH/25 g body weight continuously administered *icv* over 14 days before mice were euthanized and non-fasting blood collected.[5] The MILLIPLEX® MAP kit with 6-plex mouse metabolic magnetic bead panel was used to quantitate leptin, insulin, glucagon, GLP-1, IL-6, and TNFα in these non-fasting samples. The leptin and insulin levels were published previously[5] and used in Study 2 for comparing ELISA and LUMINEX assays. In Study 3, the non-fasting plasma insulin, glucagon, GLP-1, IL-6, and TNFα levels were analyzed for association with reversal of an obese phenotype.

Statistics

GraphPad Prism v7 software (GraphPad Software, San Diego, CA, USA) was used to perform statistical analyses. Significant differences between treatment groups on each diet were determined using two-way ANOVA and Tukey's posthoc analysis. Pearson's correlation analysis was used to examine the

relationship between glucagon and GLP1 or IL6 and TNFα. For statistical tests, a probability level of $p < .05$ was regarded as significant. All values are expressed as mean ± SEM.

Results

Plasma could not be prepared from blood that was spiked with DPPIV inhibitor, Protease inhibitor and Pefabloc

The LUMINEX protocol recommends spiking protease inhibitors into blood once collected. It was not clear why these inhibitors were recommended to be spiked after the blood was collected. We chose to spike the blood collection tubes prior to blood collection. However, when we did this we were unable to separate plasma from red cells due to hemolysis for eight samples. To overcome this problem, we collected blood into EDTA-coated microtainer tubes, prepared the plasma, and then transferred the plasma to tubes that were pre-spiked with DDPIV inhibitor, protease inhibitor, and pefabloc.

Study 1: LUMINEX assays can be performed reliably using half of the volumes specified in the manufacturer's protocol

The purpose was to determine whether LUMINEX assays can be made more cost effective by using half volumes compared to the manufacturer's protocol. Standards and two QCs provided in the single-plex adiponectin LUMINEX kit and two mouse plasma samples were compared side-by-side using either full-volume or half-volume. 'In-house' analyses for intra- and inter-assay coefficient of variation (CV) were performed using half-volumes for the assay and the two mouse plasma samples. The % CV of standard curve replicates and the Chi-Square test statistic for the distance between observed concentrations with expected concentrations, did not differ between full- and half-volume assays. The adiponectin LUMINEX % CV was similar to the in-house intra-assay % CV while the reported adiponectin LUMINEX % CV for inter-assay variation was three-fold lower than the in-house % CV (Table 2). The QCs produced adiponectin levels within the expected range and along with the plasma samples, produced similar values for half- and full-volume assays (Table 3). Therefore, the half-volume single-plex adiponectin LUMINEX assay proved to be a reliable assay.

A similar comparison between full- and half-volume assays was performed using the 6-plex LUMINEX assay (Table 2 and Table 4). When standards, two QCs and two plasma samples were compared side-by-side, the half-volume assay standard curves produced linearity that was as good as the full-volume assay. 'In-house' analyses for intra- and inter-assay CV were performed for the

Table 2. Comparison between manufacturer's and In-house intra- and inter-assay % CVs. The ELISA manufacturer's reported % CV for intra-and inter-assay variation are shown. The LUMINEX assay manufacturer's reported % CV for intra-and inter-assay variation using full-volume samples are shown alongside in-house determined intra- and inter-assay % CV using half-volume samples. 'In-house' analyses for intra- and inter-assay coefficient of variation were performed for the analytes measured in single-plex and 6-plex LUMINEX assays using half-volumes for the assay and two mouse plasma samples (described in Table 3).

Assay	Manufacturer's mean intra-assay % CV[&]	Manufacturer's mean inter-assay % CV[&]	In-House mean intra-assay % CV[++]	In-House mean inter-assay % CV[++]
ELISA				
Leptin	2	4		
Insulin	8	18		
LUMINEX				
Adiponectin	3	11	5[@]	5[$]
Leptin	5^	7*	10[+]	21[#]
Insulin	5^	7*	6[+]	21[#]
Glucagon	13^	10*	16[+]	24[#]
IL-6	5^	9*	15[+]	14[#]
TNFα	6^	5*	14[+]	15[#]
GLP-1	5^	12*	4[+]	22[#]

&Manufacturer's LUMINEX assay performed with 10 µL sample (full volume)
++In-house LUMINEX assay performed with 5 µL sample (half volume)
^Calculated from 7 replicates and 2 different cytokine concentrations in a single assay (described in LUMINEX kit protocol)
*Calculated from 6 different assays for 2 different cytokine concentrations (described in LUMINEX kit protocol)
+Calculated for 8 replicates of QC1 (provided in LUMINEX kit)
#Calculated for 3 different assays for QC1 (provided in LUMINEX kit)
@Calculated from 10 replicates of QC1 (provided in LUMINEX kit)
$Calculated from 3 different assays for QC1 (provided in LUMINEX kit)

Table 3. Comparison of adiponectin concentrations measured using half-volume and full-volume single-plex adiponectin LUMINEX assay. The QCs produced adiponectin levels within the expected range (according to LUMINEX protocol) and along with the plasma samples, produced similar values for half- and full-volume assays. The plasma samples were taken from a WT lean mouse and a *Pomc*$^{tm1/tm1}$ obese mouse to provide different measurements of the metabolic spectrum.

QC or plasma sample	Adiponectin assayed with half-volume (pg/mL)	Adiponectin assayed with full-volume (pg/mL)	LUMINEX expected range for Adiponectin (pg/mL)
QC1	493	327	233–484
QC2	4302	3295	2914–6052
Plasma 1 (Female WT, LF diet, fasting)	2408	2796	
Plasma 2 (Male *Pomc*$^{tm1/tm1}$, HF diet, fasting)	3637	2971	

analytes using half-volumes for the assay and the two mouse plasma samples. The % CV of standard curve replicates and the Chi-Square test statistic for the distance between observed concentrations with expected concentrations, did not differ between full- and half-volume assays. The leptin, insulin, glucagon, GLP-1, IL-6, and TNFα reported LUMINEX % CV ranged from being similar to three-fold lower than the in-house intra-assay and inter-assay variations (Table 2). The QCs assayed using half volumes produced IL-6, insulin, and TNF-α values within the expected manufacturer's ranges, while glucagon and

Table 4. Comparison of 6-plex LUMINEX mouse metabolic bead panel assay data using half-volume produces QC analyte (provided with assay kit) values in, or close to, the expected LUMINEX range (according to LUMINEX protocol). The plasma samples were taken from a WT lean mouse and a *Pomc*$^{tm1/tm1}$ obese mouse to provide different measurements of the metabolic spectrum.

QC or Plasma sample	Analyte in 6-plex	Analyte assayed with half-volume (pg/mL)	LUMINEX expected range for Analyte (pg/mL)
QC1	GLP-1	1797	775–1610
	IL-6	544	483–1003
	Glucagon	194	246–511
	Insulin	1386	1173–2436
	Leptin	1126	1238–2571
	TNFα	661	407–846
QC2	GLP-1	4663	2211–4592
	IL-6	1727	1467–3048
	Glucagon	806	851–1767
	Insulin	4000	3582–7439
	Leptin	3765	3678–7640
	TNFα	1648	1189–2468
Plasma 1 (Female WT, LF diet, fasting)	GLP-1	Undetected	
	IL-6	18	
	Glucagon	Undetected	
	Insulin	Undetected	
	Leptin	225	
	TNFα	Undetected	
Plasma 2 (Male *Pomc*$^{tm1/tm1}$, HF diet, fasting)	GLP-1	115	
	IL-6	256	
	Glucagon	159	
	Insulin	Undetected	
	Leptin	2438	
	TNFα	Undetected	

leptin produced values slightly below, and GLP-1 produced values slightly above, the expected manufacturer's range (Table 4). The half-volume assays passed the QC tests for high or low bead counts. Therefore, the half-volume 6-plex LUMINEX assay proved to be a reliable assay.

Study 2: LUMINEX quantitation for leptin levels is reduced compared with ELISA but LUMINEX quantitation for insulin levels is increased compared with ELISA

Good correlation between LUMINEX and ELISA data for leptin, but LUMINEX values are lower for leptin compared with ELISA values

Fasting mouse plasma leptin measured using an ELISA was previously published for male and female WT and *Pomc*$^{tm1/tm1}$ mice fed either low-fat or high-fat diet.[5] The same plasma samples were assayed using a 6-plex LUMINEX assay kit. The ELISA and LUMINEX data were correlated separately for fasting male, fasting female, fasting male plus fasting female, non-fasting male, and fasting plus non-fasting male mice. Pearson's correlation analysis showed that leptin ELISA and LUMINEX values were positively

correlated in 17 different fasting male plasma (r = 0.9645; $P < .0001$) (Figure 1a) and 28 different fasting female plasma (r = 0.9606; $P < .0001$) (Figure 1b). Linear regression ($F_{(1,26)} = 310.3$; $P < .0001$; Y = 0.3626*X – 0.05945) and Bland-Altman analysis (Bias ± SD = −1.231 ± 1.196) of the female and male ($F_{(1,15)} = 199.8$; $P < .0001$; Y = 0.4334*X + 0.3404) and (Bias ± SD = −1.532 ± 2.742) data showed that LUMINEX gives lower values compared with ELISA. When fasting female and male data were pooled, Pearson's correlation analysis again showed that leptin ELISA and LUMINEX values were positively correlated (r = 0.9531; $P < .0001$) while linear regression ($F_{(1,43)} = 409$; $P < .0001$; Y = 0.4370*X + 0.00219) and Bland-Altman analysis (Bias ± SD = −0.4217 ± 1.634) again showed that LUMINEX gives lower values for leptin compared with ELISA (Figure 1c).

Non-fasting mouse plasma leptin measured using ELISA was also previously published for male *Pomc*$^{tm1/tm1}$ mice fed either LF or HF diets and treated with vehicle, α-MSH, or desacetyl-α-MSH to reverse obesity.[5] Here, these same plasma samples were assayed using a 6-plex LUMINEX assay kit. Pearson's correlation analysis of ELISA and LUMINEX data for male non-fasting leptin levels showed that leptin values were relatively weakly positively correlated (r = 0.3374; $P = .0310$) for 41 samples compared with fasting leptin values (Figure 2a versus Figure 1a). Similar to fasting leptin values, linear regression ($F_{(1,39)} = 5.012$; $P = .0310$; Y = 0.1090*X + 2.388) and Bland-Altman analysis (Bias ± SD = −5.668 ± 8.393) data showed that LUMINEX values for non-fasting leptin were lower compared with ELISA values (Figure 2a). When fasting and non-fasting male leptin levels were pooled, Pearson's correlation analysis showed that leptin ELISA and LUMINEX values were weakly positively correlated (r = 0.4707; $P = .0002$) and linear regression ($F_{(1,56)} = 15.94$; $P = .0002$; Y = 0.1566*X + 1.752) and Bland-Altman analysis (Bias ± SD = −12.230 ± 8.191) data showed that LUMINEX gives lower values for leptin compared with ELISA (Figure 2b).

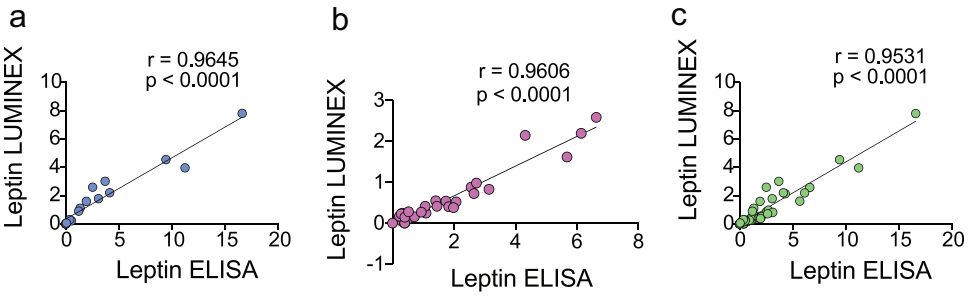

Figure 1. Fasting plasma leptin levels quantitated by LUMINEX assay and ELISA are positively correlated. Pearson's correlation shown for fasting leptin levels for male (a; n = 17), female (b; n = 28), and male plus female combined (c; n = 45). r and *P* values are provided within each graph.

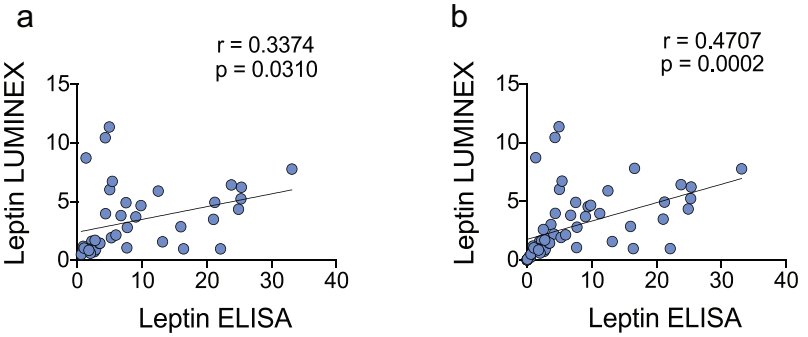

Figure 2. Non-fasting plasma leptin levels quantitated by LUMINEX assay and ELISA are positively correlated. Pearson's correlation shown for non-fasting leptin levels for male (a; n = 41) and fasting (see Figure 1a) plus non-fasting male combined (b; n = 58) leptin levels. r and *P* values are provided within each graph.

Good correlation between LUMINEX and ELISA data for insulin, but LUMINEX values are higher for insulin compared with ELISA values

Fasting mouse plasma insulin measured using an ELISA were previously published for male, but not female, WT, and *Pomc*$^{tm1/tm1}$ mice fed either LF or HF diets.[5] This was because insulin levels were detected using ELISA in only 3/21 female versus 21/21 male mouse plasma obtained from WT or *Pomc*$^{tm1/tm1}$ mice fed either LF or HF diets. Despite this, fasting male and female WT and *Pomc*$^{tm1/tm1}$ plasma samples were assayed using a 6-plex LUMINEX assay kit and the data were correlated with the ELISA data. Fasting insulin levels were detected using LUMINEX in 2/21 female and 5/21 male mouse plasma. While Pearson's correlation analysis showed that insulin ELISA and LUMINEX values were positively correlated in male plasma (r = 0.9922; $P < .0001$) (Figure 3a) and female plasma (r = 0.9526; $P < .0001$) (Figure 3b), the female data were contingent on one data point and hence unreliable. Linear regression ($F_{(1,19)} = 1204$; $P < .0001$; Y = 2.545*X – 0.4454) and Bland-Altman analysis (Bias ± SD = −0.0579 ± 0.329) of the male data showed that LUMINEX gives higher values compared with ELISA. When female and male data were pooled (n = 42), Pearson's correlation analysis showed that similar to the analysis for male data, insulin ELISA and LUMINEX values were positively correlated (r = 0.9198; $P < .0001$) (Figure 3c) while linear regression ($F_{(1,40)} = 220$; $P < .0001$; Y = 1.881*X – 0.1248) and Bland-Altman analysis (Bias ± SD = 0.023 ± 0.356) showed that LUMINEX gives higher values for insulin compared with ELISA.

In contrast to fasting insulin, ELISA or LUMINEX proved suitable for quantitating non-fasting mouse insulin for male *Pomc*$^{tm1/tm1}$ mice fed either LF or HF diets and treated with vehicle, α-MSH, or desacetyl-α-MSH to reverse obesity (Figure 4a). The ELISA data showed α-MSH significantly reversed male *Pomc*$^{tm1/tm1}$ high-fat diet-induced hyperinsulinemia (Figure 4a). Here, these same plasma samples were assayed using a 6-plex LUMINEX assay kit. Insulin

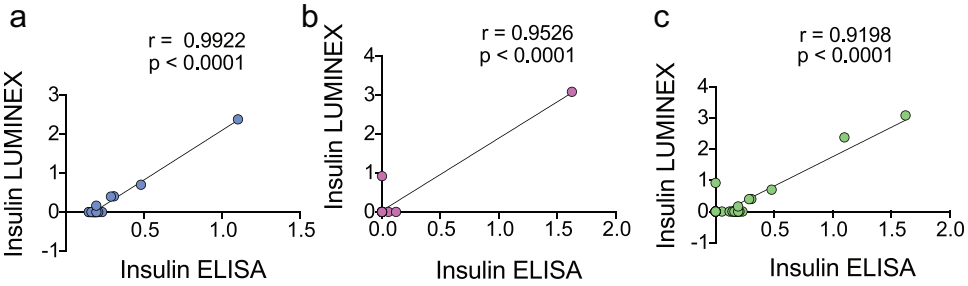

Figure 3. Fasting plasma insulin levels quantitated by LUMINEX assay and ELISA are positively correlated. Pearson's correlation shown for fasting insulin levels for male (a; n = 21), female (b; n = 21) and male plus female combined (c; n = 42). r and *P* values are provided within each graph.

was detected in 44/45 of non-fasting male mouse plasma samples using either ELISA or the LUMINEX assay, and the quantitative data obtained were similar between these assays. Pearson's correlation analysis of ELISA and LUMINEX data for male non-fasting insulin levels showed that insulin values were positively correlated (r = 0.9643; $P < .0001$) (Figure 4b). Similar to fasting insulin values, linear regression ($F_{(1,43)} = 571$; $P < .0001$; Y = 0.9582*X + 0.1295) and Bland-Altman analysis (Bias ± SD = 0.079 ± 0.474) data showed that LUMINEX values for non-fasting insulin were higher compared with ELISA values (Figure 4b). When fasting and non-fasting male insulin levels were pooled, Pearson's correlation analysis showed that insulin ELISA and LUMINEX values were positively correlated (r = 0.9610; $P < .0001$) (Figure 4c). Linear regression ($F_{(1,64)} = 772.4$; $P < .0001$; Y = 0.9830*X + 0.05084) and Bland-Altman analysis (Bias ± SD = 0.036 ± 0.435) data showed that LUMINEX gives higher values for insulin compared with ELISA.

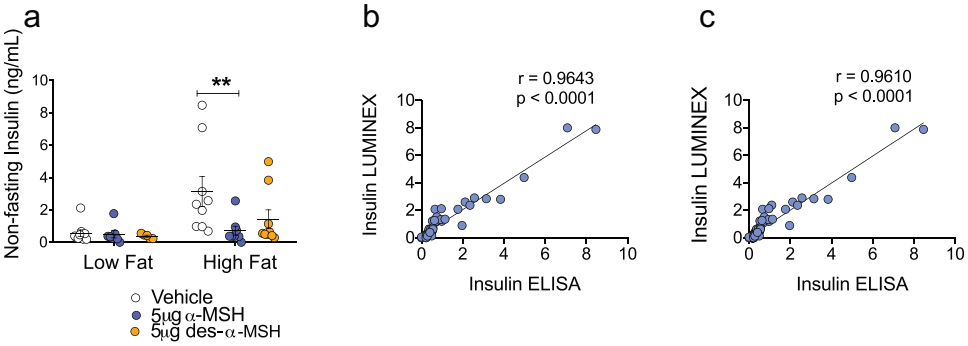

Figure 4. Non-fasting plasma insulin levels quantitated by ELISA and LUMINEX assays. a, ELISA data show *icv* α-MSH treatment significantly reverses male *Pomc*$^{tm1/tm1}$ mouse HF diet-induced hyperinsulinemia. Two-way ANOVA and Tukey's posthoc multiple comparison test show α-MSH significantly reduces insulin levels. **, $p < .01$. band c, LUMINEX data are positively correlated with ELISA. Pearson's correlation shown for non-fasting insulin levels for male (b; n = 45) and fasting (see Figure 3a) plus non-fasting male combined (c; n = 66) insulin levels. r and *P* values are provided within each graph.

Study 3: The 6-plex LUMINEX mouse metabolic magnetic bead panel lacks sensitivity to quantitate most fasting and non-fasting mouse plasma for glucagon, GLP, IL-6 and TNFα

Glucagon and GLP-1

Glucose homeostasis depends on the coordinated secretion of insulin, glucagon, and GLP-1. Plasma glucagon and GLP-1 levels are expected to change with fasting and feeding. Fasting decreases blood glucagon levels of obese mice but increases glucagon levels of lean mice.[13] Feeding induces GLP-1 secretion from enteroendocrine cells and compared to lean individuals, obese individuals show reduced feeding GLP-1 response.[14,15] In this study, there were no trends for fasting or non-fasting glucagon or GLP-1 concentrations to associate with mouse metabolic status. This is believed to be due to the high proportion of samples with undetectable glucagon and GLP-1 levels in the LUMINEX assay.

More females than males had detectable fasting plasma glucagon (10 females, 4 males) or GLP-1 (6 females, 2 males) in a cohort of 52 mice (male plus female) (Figure 5a–d), consistent with previous findings of a sex difference for glucagon levels.[16] The plasma with detectable glucagon or GLP-1 presented with concentrations that were within the ranges for standards provided in the LUMINEX assay kit, albeit at the lower end (Table 1). Seven of the samples with detectable glucagon also had detectable GLP-1. While fasting glucagon or GLP-1 concentrations did not significantly associate with mouse metabolic status, this data is considered unreliable since 38/52 and 44/52 plasma had undetectable glucagon and GLP-1, respectively.

Non-fasting plasma glucagon and GLP-1 levels were detected for only eight and three male mouse samples, respectively, out of a total of 45 samples measured using the 6-plex LUMINEX assay (Figure 6a,b). This data is not considered reliable to determine whether non-fasting glucagon or GLP-1 concentrations associate with mouse obese-genotype or reversal of mouse obesity, since 37/45 and 42/45 plasma had undetectable glucagon and GLP-1, respectively. While quantitative levels for three plasma with detectable glucagon and GLP-1 positively correlated, this was dependent on one plasma and therefore unreliable (Figure 6e).

IL-6 and TNFα

Blood concentrations of cytokines are expected to be very low but increase markedly with inflammation. Evidence of a hyperinflammatory state associated with obesity and HF diet is often indicated by increased levels of proinflammatory cytokines, IL-6 and TNFα, in blood.[12,17–21] In this study there were no trends for the fasting or non-fasting IL-6 or TNFα

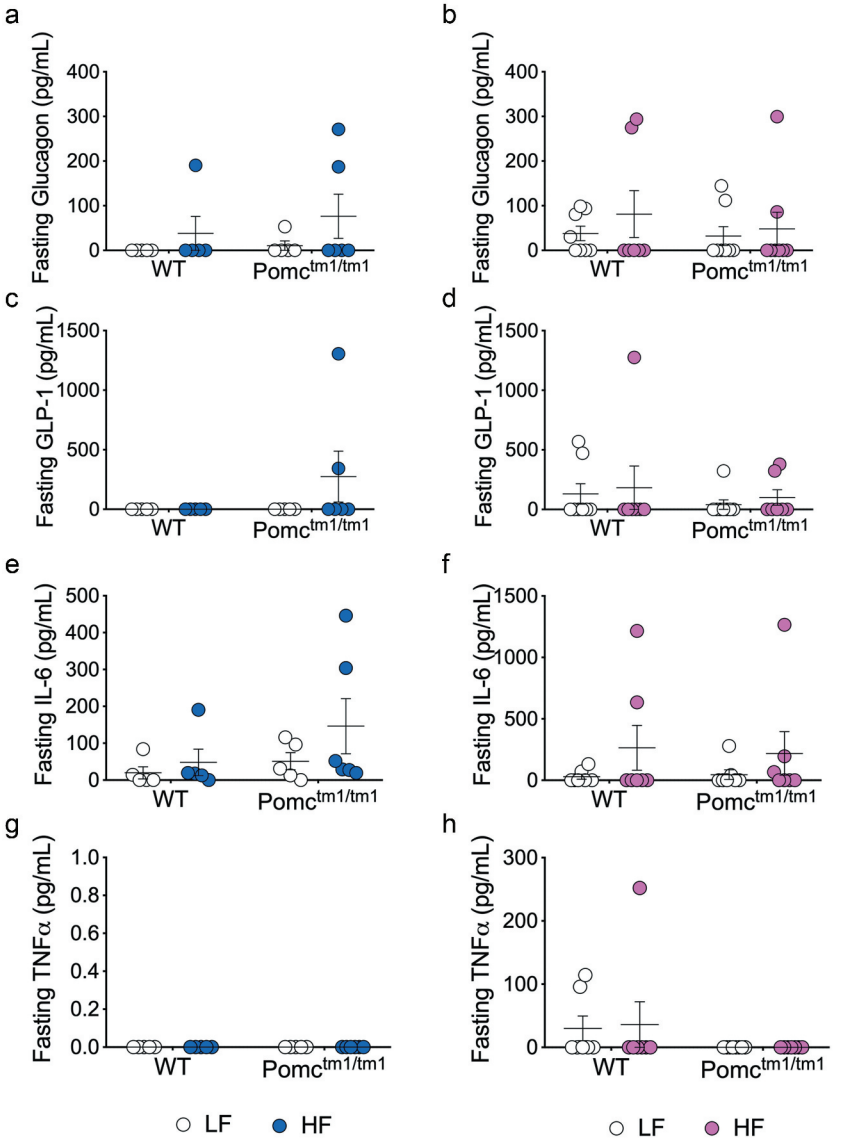

Figure 5. Fasting plasma glucagon, GLP-1, IL-6 and TNFα levels quantitated by LUMINEX assay. Plasma was obtained from male and female WT and *Pomc*$^{tm1/tm1}$ mice were euthanized following feeding on LF or HF diet for 23 weeks as described in a previous study.[5] (a),male glucagon; (b), female glucagon; (c), male GLP-1; (d), female GLP-1; (e), male IL-6; (f), female IL-6; (g), male TNFalpha; (h) female TNFalpha. Male WT fed LF diet (n = 5); male WT fed HF diet (n = 5); male *Pomc*$^{tm1/tm1}$ fed LF diet (n = 5); male *Pomc*$^{tm1/tm1}$ fed HF diet (n = 6); female WT fed LF diet (n = 8); female WT fed HF diet (n = 7); female *Pomc*$^{tm1/tm1}$ fed LF diet (n = 8); female *Pomc*$^{tm1/tm1}$ fed HF diet (n = 8). No significant differences were shown between treatment groups on each diet using two-way ANOVA and Tukey posthoc analysis.

concentrations to associate with mouse metabolic status. This is believed to be due to the high proportion of samples with undetectable IL-6 and TNFα in the LUMINEX assay.

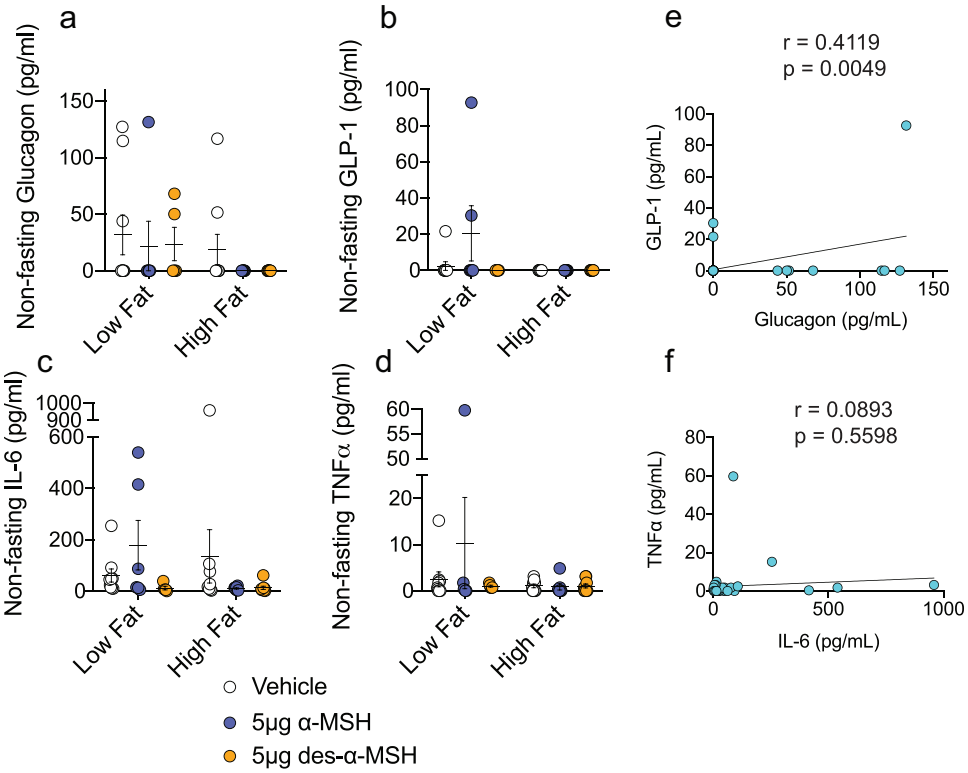

Figure 6. Non-fasting plasma glucagon, GLP-1, IL-6 and TNFα levels quantitated by LUMINEX assay. Glucagon (a), GLP-1 (b), IL-6 (c) and TNFα (d) levels were measured for plasma was obtained from male *Pomc*$^{tm1/tm1}$ mice fed LF or HF diets from weaning. At age 22 weeks mice were *icv* administered vehicle, 5 μg α-MSH/25 g body weight or 5 μg desacetyl-α-MSH/25 g body weight continuously over 14 days, and then euthanized for a previous study.[5] The groups of mice were LF diet treated with vehicle (n = 9), LF diet treated with α-MSH (n = 6), LF diet treated with desacetyl-α-MSH (n = 5), HF diet treated with vehicle (n = 9), HF diet treated with α-MSH (n = 7) and HF diet treated with desacetyl-α-MSH (n = 9). No significant differences were shown between treatment groups on each diet using two-way ANOVA and Tukey posthoc analysis. Pearson's correlation shown for non-fasting glucagon and GLP1 levels from all treatment groups combined (e; n = 45) and non-fasting IL-6 and TNFα from all treatment groups combined (f; n = 45). Pearson r and *P* values are provided within each graph.

Fasting plasma IL-6 and TNFα levels were detected for only 25 and 3 samples, respectively, out of a total of 52 samples measured using the LUMIINEX assay (Figure 5e–h). More males (16) than females (9) had detectable IL-6, which is consistent with a stronger obesity-associated inflammatory response in males compared with females.[22,23] While fasting IL-6 levels appeared increased with HF diet in male and female WT and *Pomc*$^{tm1/tm1}$ mice (Figure 5e,f), this data is unreliable since IL-6 was not detected for 27/52 plasma and 8 of the plasma with detectable IL-6 presented with concentrations below the lowest standard provided in the assay kit (27 pg/mL) (Table 1). In contrast to IL-6, TNFα was not detected in any fasting male plasma. Fasting

TNFα was only detected in three female plasma with concentrations within the standard range for the assay kit (Table 1). All three female plasma with detectable TNFα also had detectable IL-6.

Non-fasting plasma IL-6 and TNFα levels were detected for 45/45 and 29/45 male mouse samples, respectively, using the 6-plex LUMINEX assay (Figure 6c,d). The quantitative IL-6 data were highly variable and IL-6 was detected in 30/45 plasma with concentrations below the lowest standard for the assay (Figure 6c and Table1). Similarly, the non-fasting TNFα data were highly variable. TNFα was detected in 26/45 plasma with concentrations below the lowest standard for the assay (Figure 6d and Table 1). There was no correlation between non-fasting IL-6 and TNFα where 29/45 plasma had detectable IL-6 and TNFα levels (Figure 6f).

Discussion

Here, the evidence is provided that can guide investigators in the future in making decisions about which metabolic hormone and cytokine assays to employ for mouse metabolic studies. First, I show that LUMINEX assays can be made more cost-effective without compromising quality data, by halving volumes for assay compared with the manufacturer's protocol. Second, I show good correlations for fasting mouse leptin and non-fasting mouse insulin between the same samples performed in a 6-plex mouse metabolic magnetic bead panel LUMINEX assay and ELISA. Third, the LUMINEX assay was shown to be unsuitable for quantitating fasting insulin, since insulin was detected in few fasting male or female mouse plasma. Fourth, ELISA was shown to be suitable for quantitating fasting male, but not female, mouse insulin. Fifth, the concurrence of quantitative values between the LUMINEX assay and ELISA was poor for leptin and insulin. The LUMINEX assay gave lower values for leptin and higher values for insulin, compared with ELISA. Sixth, the mouse metabolic LUMINEX assay proved unsuitable for quantitating glucagon, GLP-1, IL-6, and TNFα, due to undetectable levels for most fasting and non-fasting plasma obtained from mice with a wide range of metabolic status. Overall, quantitative leptin levels were the only useful data obtained from the 6-plex mouse metabolic magnetic bead panel LUMINEX assay.

While the correlation between LUMINEX and ELISA data was good for quantitating fasting mouse leptin, the correlation was comparatively weak for non-fasting mouse leptin. The reason for the comparatively weak correlation for non-fasting leptin is unclear. Furthermore, LUMINEX gives lower values for leptin compared with ELISA assay data. Despite the quantitative differences, either multiplex LUMINEX or ELISA proved suitable for quantitating leptin levels for fasting male and female, as well as non-fasting male, C57BL/6 J mice.

The correlation between LUMINEX and ELISA data was good for quantitating fasting mouse male insulin, albeit with limited samples for which insulin was detected, and also for non-fasting male plasma insulin. In contrast with leptin, there was a relatively good concurrence between LUMINEX and ELISA quantitative values for insulin, although the LUMINEX assay gave higher values for insulin compared with ELISA. However, because of the low sample number where insulin was detected, the 6-plex metabolic bead panel LUMINEX assay proved unsuitable for quantitating fasting male or female C57BL/6 J mouse insulin. ELISA also proved unsuitable for quantitating fasting female, but not male, C57BL/6 J mouse insulin. Indeed, using ELISA quantitation of non-fasting insulin showed that *icv* α-MSH administration significantly reverses male *Pomc*$^{tm1/tm1}$ mouse hyperinsulinemia on HF diet (Figure 4a).

The use of half-volumes for single-plex and 6-plex LUMINEX assay trialed here, worked well and allowed almost 80% more samples to be quantitated in a single assay kit. Despite the relatively high inter- and intra- % CV observed 'in-house' compared with the manufacturer's data, both the single-plex LUMINEX adiponectin assay and the 6-plex LUMINEX fasting leptin assay, provided valuable data identifying significant differences between mouse metabolic states.[5] Thus, the use of half-volumes improves the cost-effectiveness for the LUMINEX assay kit. Further work is required to more accurately determine the inter-and intra- % CV when using half-volumes.

No reliable data were obtained for quantitating glucagon, GLP-1, IL-6, or TNFα levels in fasting or non-fasting male or female mice with a range of metabolic phenotypes. A potential contributing factor to this disappointing outcome was the unsuitable range of standards, and hence sensitivity for these analytes in the 6-plex LUMINEX kit for the mouse metabolic phenotypes used in this study. Quantitative levels for many of these analytes in mouse metabolic studies that have been published (Table 1) are either a lot lower, or at the low end, of the standards present in the 6-plex LUMINEX kit used here. There are other LUMINEX assays available that specifically measure cytokines with standards in the lower range. They would likely produce helpful data for mouse metabolic studies.

In conclusion, multiple LUMINEX assays are only cost- and time-effective if data obtained are informative for research and diagnostics. Here, quantitative leptin was the only useful data we obtained after using a 6-plex mouse metabolic magnetic bead panel LUMINEX assay. Therefore, this MILLIPLEX® MAP kit with 6-plex mouse metabolic magnetic bead panel was not cost-effective for this study. I learned this lesson by trialing this assay and comparing it with ELISA. Based on this experience, I offer recommendations for investigators in the future who are choosing assays to invest in, for measurement of mouse metabolic hormones and cytokines. First, if samples are to be collected that require addition of protease inhibitors to prevent analyte degradation, I recommend preliminary studies

Table 5. Recommendations for choosing between ELISA and LUMINEX assays to quantitate leptin and insulin levels for mouse metabolic studies.

Sex	Fasting/Non-Fasting	Hormone	Assay	Comment
Male and Female	Fasting	Leptin	ELISA or LUMINEX	LUMINEX values < ELISA values with good correlation between assays
Male	Non-Fasting	Leptin	ELISA or LUMINEX	LUMINEX values < ELISA values with poor correlation between assays
Male	Fasting	Insulin	ELISA	
Female	Fasting	Insulin	Ultra-Sensitive ELISA	
Male	Non-Fasting	Insulin	ELISA or LUMINEX	LUMINEX values > ELISA values with good correlation between assays

to establish a method for blood collection and sample preparation that will eliminate hemolysis prior to collecting precious samples for a large study. Second, when deciding on an assay, I recommend taking into consideration the sex of the mice and whether blood is collected from fasted or non-fasted mice. Based on the data presented here, I have summarized my recommendations for mouse leptin and insulin assays (Table 5). Third, the MILLIPLEX® MAP kit with 6-plex mouse metabolic magnetic bead panel used here proved unsuitable for providing any meaningful data on glucagon, GLP-1, IL-6, and TNFα for mouse metabolic studies. While there may be multiple reasons for this, I recommend where possible for future studies, to investigate whether the range of standards for these analytes in the LUMINEX kit appear suitable for predicted quantitation of the analytes based on previous publications by other investigators. Finally, half-volumes for the single-plex adiponectin LUMINEX assay were adequate to produce reliable data. Therefore, I recommend consideration be given to cost-effectiveness of using LUMINEX assays with half-volumes compared with the manufacturer's protocol.

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Disclosure statement

I declare that I have no conflicts of interest with the content of this article.

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