

# Development and Validation of Multiplexed Metabolic Assay Panel Using ECL Technology

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## PURPOSE

The increased prevalence of metabolic-related diseases including diabetes, obesity, and metabolic syndrome has driven demand for the sensitive measurement of biomarkers linked to these disease states. The expression levels of these biomarkers, produced in the gut, adipose tissue, and brain, are frequently altered during disease and can be measured in plasma and serum. To address the need for sensitive and validated biomarker assays, we developed individual and multiplexed assays targeting seven metabolic biomarkers for use in human, nonhuman primate, canine, and rodent samples. The metabolic panel consists of assays for Insulin, C-peptide, GLP-1 Active (7-36 amide), GIP Active (1-42), Glucagon, PP, and Leptin. Using this panel, endogenous analyte levels can be measured and monitored throughout the metabolic process with as little as 25 µL of sample.

## METHODS

MSD's electrochemiluminescence detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® and MULTI-SPOT® microplates.



### Electrochemiluminescence Technology

- Minimal non-specific background and strong responses to analyte yield high signal-to-background ratios.
- The stimulation mechanism (electricity) is decoupled from the response (light signal), minimizing matrix interference.
- Only labels bound near the electrode surface are excited, enabling non-washed assays.
- Labels are stable, non-radioactive, and directly conjugated to biological molecules.
- Emission at ~620 nm eliminates problems with color quenching.
- Multiple rounds of label excitation and emission enhance light levels and improve sensitivity.
- Carbon electrode surface has 10X greater binding capacity than polystyrene wells.
- Surface coatings can be customized.

Challenges associated with multiplexing were solved through epitope mapping, extensive antibody screening followed by antibody engineering to enhance sensitivity and specificity, and substantial diluent optimization to reduce matrix effects. Calibrators were anchored to in-house or industry-accepted reference standards. Analytical validation was performed across multiple lots and included testing of dynamic range, matrix tolerance, reproducibility, and limits of quantitation (LOQ). Specificity was tested against common metabolic markers and their structurally related analogs and fragments. Assay robustness was confirmed by varying temperatures and incubation times, and testing of other common use-case scenarios including the presence of interfering agents.

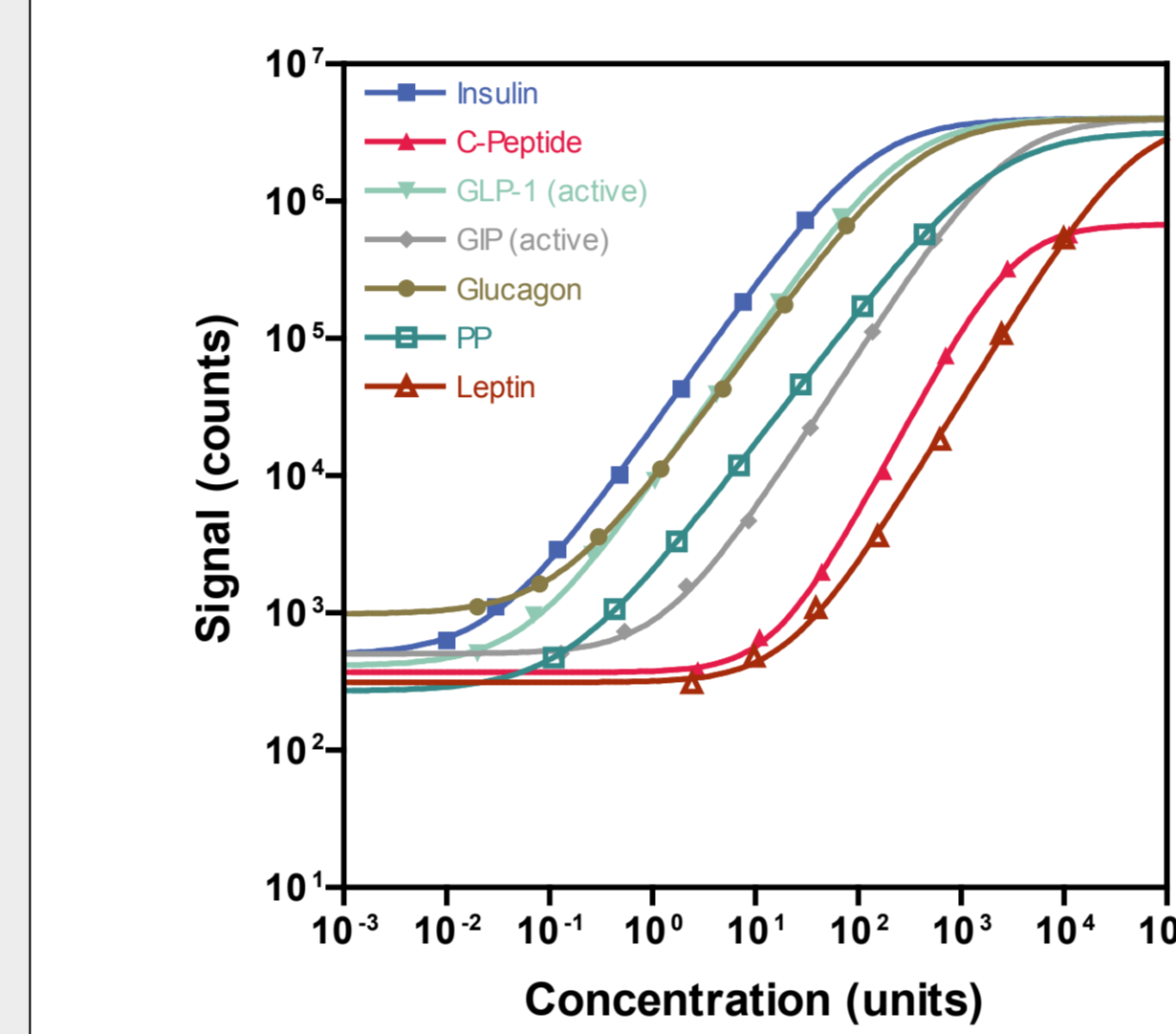
## RESULTS

We developed assays for seven metabolic markers that are highly specific and demonstrate little interference from other blood components. These assays have dynamic ranges of over three logs and best-in-class sensitivities with lower limits of quantitation (LLOQ) for key metabolic assays such as Insulin, Glucagon, and GLP-1 Active (7-36 amide) (0.07 µIU/mL, 0.33 pM, and 0.10 pM, respectively). These highly sensitive assays permit accurate quantitation even in populations with suppressed secretion such as fasting, diabetic, or heart disease groups. Accuracy and precision were confirmed by testing three levels of controls across multiple lots, and all controls had concentration intra-run and inter-run %CVs of less than 7% with 80-120% recovery of target concentrations. Matrix compatibility was verified through parallelism, dilution linearity, and spike recovery studies in serum or plasma treated with protease inhibitors. Owing to high cross-species homology for several analytes on the panel, matrix studies included multiple species. These studies demonstrated matrix tolerance and accurate quantitation for most of the seven assays (typically between 80-120%). Most of the seven metabolic assays exhibited minimal cross-reactivity and interference from analytes that share significant sequence homology.

## CALIBRATION CURVES AND LIMITS OF DETECTION

The figure to the right demonstrates typical calibration curves for the analytes in the V-PLEX® Metabolic Kits. Data in the table include upper limit of quantitation (ULOQ), lower limit of quantitation (LLOQ), and ranges for the lower limits of detection (LLOD, n=3 runs each from a different kit lot). Calibrators were reconstituted and diluted serially (4-fold) to generate a 7-point standard curve for each panel.

Figure 1. Typical calibration curves for the V-PLEX Metabolic Kit.



Analyte	Assay Units	Median LLOD	LLOD Range	LLOQ	ULOQ
Insulin	µIU/mL	0.006	0.002-0.052	0.07	19.5
C-Peptide	pg/mL	4.72	2.07-10.2	19	3,150
GLP-1 (active)	pM	0.011	0.005-0.027	0.1	39
GIP (active)	pg/mL	0.233	0.106-0.691	3.18	390
Glucagon	pM	0.015	0.007-0.083	0.33	52
PP	pg/mL	0.043	0.022-0.212	0.41	325
Leptin	pg/mL	5.78	3.10-14.3	37.7	7,150

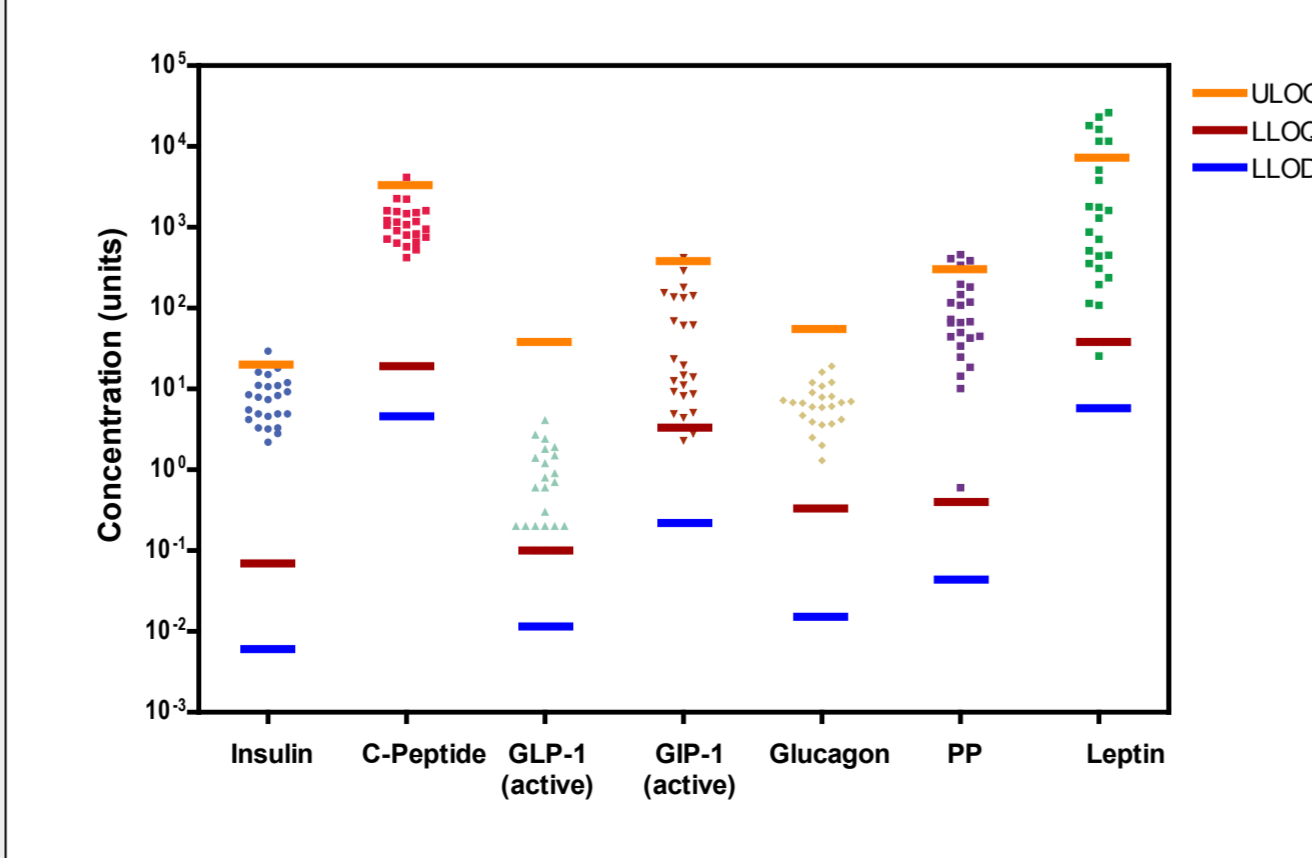
The LLOD is a calculated concentration corresponding to the average signal 2.5 standard deviations above the background (zero calibrator). The ULOQ and LLOQ are established for each lot by measuring multiple levels near the expected LLOQ and ULOQ levels. The final LLOQ and ULOQ specifications for the product are established after assessment of all validation lots.

## SAMPLE TESTING

To assess the performance of the V-PLEX assays, human samples of P800-collected EDTA plasma from apparently healthy individuals obtained from a commercial source were diluted 2-fold and tested on the V-PLEX Metabolic Kit.

Plasma samples were collected from different individuals at different fasting time points (2.0 – 14.5 hours). The sample concentration prior to dilution factor adjustment is displayed; see Figure 2, below. The results are in agreement with the expected concentrations reported in the literature and the majority of samples contained analyte levels above the LLOQ of each assay.

Figure 2. Fasting levels of metabolic analytes in individuals at different time points, measured using the V-PLEX Metabolic Panel Kit.



## SPECIFICITY

To assess the specificity of each assay, the V-PLEX Metabolic Kit was tested for nonspecific binding to the following proglucagon metabolites and other general metabolic targets at 1,000 pg/mL. Cross-reactivity below the assay limit of quantitation is reported as less than LLOQ (< LLOQ).

In addition, cross-reactivity to the following analytes was below the LLOQ and are not included in the table below: mouse leptin, rat leptin, canine leptin, PYY (1-36), PYY (3-36), PYY (3-34), NPY, Resistin, IL-6, Leptin receptor, ApoJ, A2M, G-CSF, LIF, Oncostatin M, CNTF, IL-11, IL-12, Ghrelin (active), and Ghrelin (inactive).

Analyte	Metabolite/Hormone	Insulin	C-Peptide	GLP-1 (active)
GLP-1 Fragments	GLP-1 (1-36)	< LLOQ	< LLOQ	< LLOQ
	GLP-1 (1-37)	< LLOQ	< LLOQ	< LLOQ
	GLP-1 (7-37)	< LLOQ	< LLOQ	73.5%
	GLP-1 (9-36)	< LLOQ	< LLOQ	< LLOQ
GIP Fragments and Mutants	GIP (1-30)	< LLOQ	< LLOQ	< LLOQ
	GIP (3-42)	< LLOQ	< LLOQ	< LLOQ
GLP-1 Receptor Agonists	Pro(3) GIP	< LLOQ	< LLOQ	< LLOQ
	Liraglutide*	< LLOQ	< LLOQ	0.16%
	Exenatide	< LLOQ	< LLOQ	< LLOQ
Proglucagon Metabolites	MPGF†	< LLOQ	< LLOQ	0.43%
	GRPP	< LLOQ	< LLOQ	< LLOQ
	Glicentin (1-61)‡	< LLOQ	< LLOQ	< LLOQ
	Glicentin (1-69)	< LLOQ	< LLOQ	< LLOQ
	GLP-2	< LLOQ	< LLOQ	< LLOQ
	Oxyntomodulin	< LLOQ	< LLOQ	< LLOQ
	Miniglucagon	< LLOQ	< LLOQ	< LLOQ
Proinsulin Fragments and Receptors	Proinsulin	0.03%	32.4%	< LLOQ
	Soluble Insulin receptor	< LLOQ	< LLOQ	< LLOQ
	IGF-1	< LLOQ	< LLOQ	< LLOQ
	IGF-2	< LLOQ	< LLOQ	< LLOQ
	Lispro Insulin	0.10%	< LLOQ	< LLOQ
Aspart Insulin	0.72%	< LLOQ	< LLOQ	
Glargine Insulin	0.76%	< LLOQ	< LLOQ	

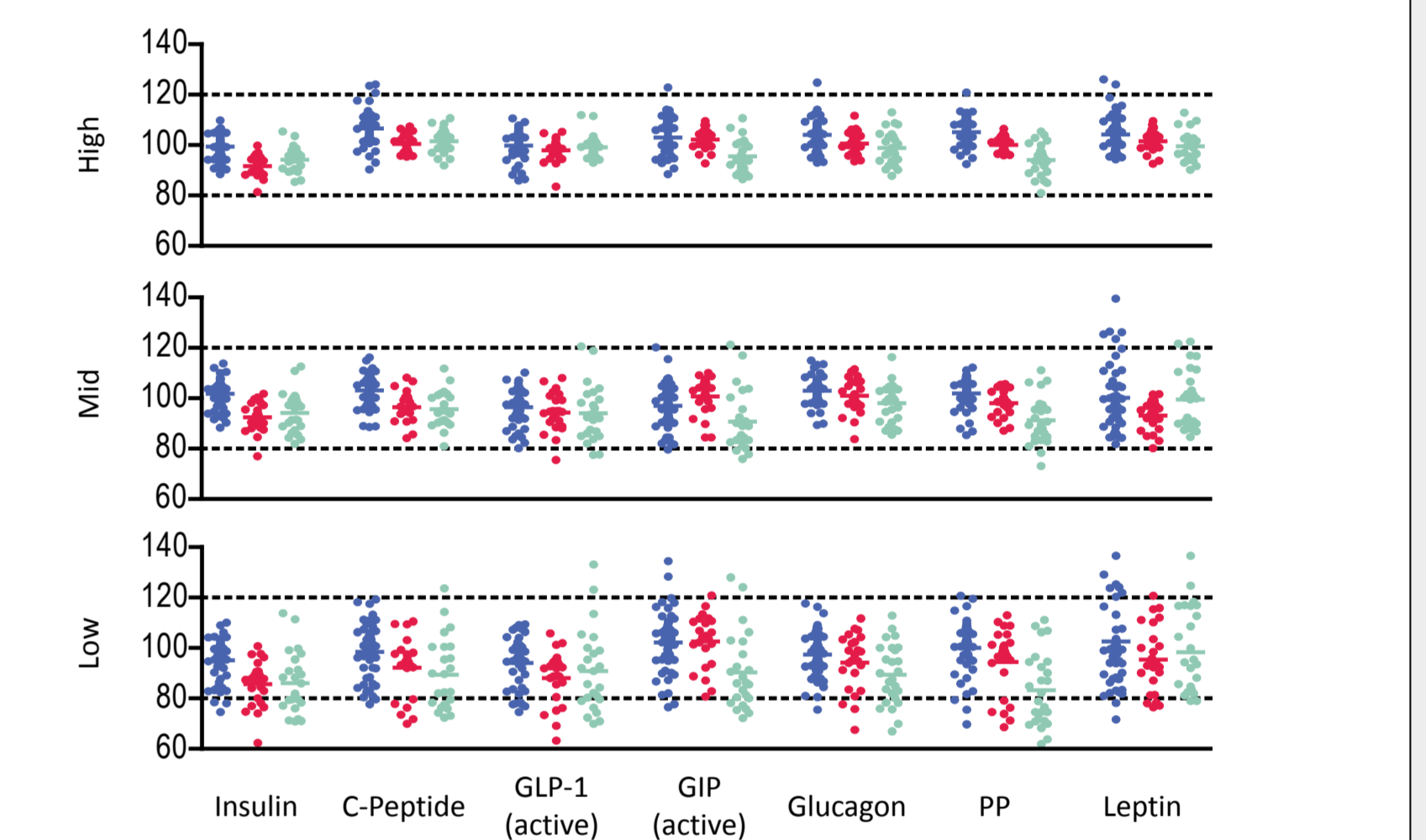
\*GLP-1 (active) concentrations may be suppressed in the presence of Liraglutide concentrations that are higher than 50 pM. †Cross-reactivity of Glucagon and circulating glicentin (1-61) is expected due to sequence similarities. ‡Major Proglucagon Fragment

## ACCURACY AND PRECISION

Quality control samples were prepared by spiking calibrator into a serum-free matrix at three levels (high, mid, and low) within the quantitative linear range of the assay. The controls were measured using a minimum of three replicates tested over multiple days and multiple operators for a total of at least 48 runs. The accuracy of control determinations fell within 20% of the expected concentration with precision of less than 20% CV in the majority of runs (Figure 3, below).

Accuracy is defined as the average measured control concentration for a given lot divided by the expected control concentration. The accuracy shown is an average of three replicates on a single plate. Runs were conducted using three kit lots by at least four different operators.

Figure 3. Control accuracy across multiple kit lots. Kit 1 (blue), Kit 2 (red), Kit 3 (green).



- Average intra-run %CV is the average %CV of the control replicates within an individual run across 48 runs (three kit lots).
- Inter-run %CV is the variability of controls across 12 runs within a single kit lot (across three kit lots).
- Inter-lot %CV is the variability of controls across 3 kit lots (total of 48 runs).

Analyte	Control Precision								
	High Control			Mid Control			Low Control		
	Intra-Run % CV	Inter-Run % CV	Inter-Lot % CV	Intra-Run % CV	Inter-Run % CV	Inter-Lot % CV	Intra-Run % CV	Inter-Run % CV	Inter-Lot % CV
Insulin	2.80	2.50	6.20	3.00	2.70	8.50	6.10	4.90	12.40
C-Peptide	3.10	2.90	6.50	2.10	1.90	6.80	3.90	3.60	12.90
GLP-1 (active)	4.00	3.80	6.30	4.50	4.20	9.20	5.80	5.20	12.70
GIP (active)	3.80	4.00	6.90	4.00	3.80	10.50	4.30	4.70	13.30
Glucagon	3.50	3.10	5.80	3.50	2.80	6.70	5.60	4.40	11.20
PP	2.80	2.90	6.20	2.40	2.30	8.40	6.20	5.40	15.20
Leptin	3.00	2.70	7.00	3.40	3.10	13.80	6.70	6.30	19.90

## CONCLUSIONS

The V-PLEX Metabolic Panel 1 assays are highly specific, sensitive, and validated for use with serum and plasma from multiple species. Multi-lot analytical validation demonstrated consistent assay performance and accurate measurements of biomarkers associated with diabetes, obesity, and metabolic syndrome, making these assays valuable tools for basic research and preclinical studies. These metabolic assays include Insulin, GLP-1 Active (7-36 amide), and Glucagon assays with best-in-class sensitivity and specificity.



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## MATRIX PERFORMANCE

### PARALLELISM, DILUTION LINEARITY, AND SPIKE RECOVERY

Serum and plasma samples collected in P800 tubes from apparently healthy human donors were obtained from a commercial source. Owing to the sequence homology of multiple analytes in the panel across mammalian species, several common animal models were also assayed. Results from mouse, non-human primate (NHP), rat, and canine samples are similar to that on human samples (data not shown).

To assess parallelism, samples with high endogenous levels of analytes were diluted 2-fold, 4-fold, 8-fold, and 16-fold, before testing. Percent recovery at each dilution level was normalized to the 2-fold dilution-adjusted concentration.

Sample Type	FOLD Dilution	Insulin		C-Peptide		GLP-1 (active)		GIP (active)		Glucagon		PP		Leptin	
		Ave. % Recovery	Range	Ave. % Recovery	Range	Ave. % Recovery	Range	Ave. % Recovery	Range	Ave. % Recovery	Range	Ave. % Recovery	Range	Ave. % Recovery	Range
Serum (n = 12)	4	107	100-114	114	85-174	131	124-137	149	127-182	109	97-124	103	90-119	119	82-189
	8	105	96-113	118	98-179	<LLOQ	NA	215	172-257	106	94-118	100	86-121	139	59-231
	16	107	98-117	119	96-181	<LLOQ	NA	<LLOQ	NA	112	93-128	100	84-118	184	59-337
P800 EDTA Plasma (n = 17)	4	112	103-140	108	97-119	107	98-123	110	93-122	111	99-117	99	92-112	127	95-170
	8	116	103-170	112	95-129	100	90-114	113	85-151	113	100-126	98	89-119	161	105-297
	16	119	104-184	119	99-138	100	89-116	103	78-122	115	104-127	99	82-128	177	97-421

To assess linearity, samples were spiked with calibrator and diluted 2-fold, 4-fold, 8-fold, and 16-fold, before testing. Percent recovery at each dilution level was normalized to the 2-fold dilution-adjusted concentration. The average percent recovery is based on samples within the quantitative range of the assay (below).

Sample Type	FOLD Dilution	Insulin		C-Peptide		GLP-1 (active)		GIP (active)		Glucagon		PP		Leptin	
		Ave. % Recovery	Range	Ave. % Recovery	Range	Ave. % Recovery	Range	Ave. % Recovery	Range	Ave. % Recovery	Range	Ave. % Recovery	Range	Ave. % Recovery	Range
Serum (n = 12)	4	107	100-115	109	97-126	120	109-131	119	106-141	104	95-121	99	85-110	114	90-129
	8	105	100-112	109	91-128	115	103-127	132	118-165	105	95-118	96	83-104	128	74-167
	16	106	99-115	113	92-140	109	98-120	139	120-184	107	91-124	94	80-105	142	68-246
P800 EDTA Plasma (n = 12)	4	108	104-114	112	102-123	101	95-111	106	92-121	111	102-120	101	97-111	128	100-174
	8	108	100-124	113	89-130	91	84-99	106	85-148	109	91-122	99	89-116	156	97-249
	16	108	98-126	119	104-140	85	80-94	105	80-147	114	95-128	101	91-123	166	89-276

To assess recovery, samples were spiked with calibrator at three levels (high, mid, and low) then diluted 2-fold. The average % recovery for each sample type is reported along with %CV and % recovery range.

Sample Type	Spike Recovery							
	% Recovery	Insulin	C-Peptide	GLP-1 (active)	GIP (active)	Glucagon	PP	Leptin
Serum (n = 12)	Average	101	86	65	40	96	98	58
	%CV	4.8	3.5	15.8	10.9	11.5	15.5	34.4
	Range	96-106	82-93	44-93	28-64	80-109	78-125	35-118
P800 EDTA Plasma (n = 12)	Average	91	78	113	86	93	92	65
	%CV	7.1	12.3	12.6	18.7	9.3	12.1	18.1
	Range	77-98	56-97	89-134	69-108	77-104	74-109	45-101