Application Note

Specific Detection of Full-length Glucagon From Blood Samples Using a Chemiluminescent ELISA Kit

Introduction

Glucagon is the most important counter-regulatory hormone to insulin in blood glucose homeostasis. It elevates blood glucose levels by stimulating gluconeogenesis and glycogenolysis while inhibiting glycogenesis and glycolysis mainly in the liver[1]. Recently, glucagon and its receptors have been actively pursued as therapeutic target for diabetes, especially Type I diabetes[2]. Therefore, it is crucial to have available an assay for accurately, precisely and rapidly quantifying glucagon in blood samples.

Normal glucagon levels are very low in healthy individuals (< 200 pg/mL) compared to glucagon levels in severe pathophysiological states, which are highlighted (Table 1).

The vast majority of glucagon levels reported in published literature are measured by radioimmunoassay (Table 1) which still remains the most popular assay for glucagon. However, due to considerations of radioactive waste management and safety, ELISAs can be performed with far greater convenience and throughput.

Method	Sample	Population Studied	No of Subjects	Measured Glucagon (pg/mL)	Ref. No.
RIA	Fasting Plasma	Type I Diabetic	12	118 ± 9 (70 ~ 200)	3
		Diabetic (with Severe Ketoacidosis)	8	587 ± 195 (230 ~ 2,000)	
		Diabetic (with Mild Ketoacidosis)	4	185 ± 13 (165 ~ 225)	
		None Obese Normal	28	108 ± 10 (50 ~ 210)	
RIA	Plasma	Obese Not Fasted	15	73 ± 4.7	4
		Obese Fasted for 3 Days		144 ± 15.7	
RIA	Plasma	Normal	12	102 ± 25	5
RIA	Fasting Plasma	Healthy Volunteers	30	87.9 ± 23.8	6
RIA	Serum	Aorto-coronary By-pass Patients	78	72.6 ± 50.8	7
RIA	Plasma	Normal Male	15	130 ± 30	8
Unknown	Plasma	Basal (Male)	9	20.9	9
		After Insulin Infusion		83.6	
RIA	Plasma	Normal Male	6	156.6 ± 17.3	10
RIA	Plasma	False Positive Case Report	1	23 ~ 197	11
				320 ~ 430 (Glucagon + OXM)	
Unknown	Plasma	Case Report, Confirmed Glucagonoma (Male)	1	1,280 (Cited Nomal Range < 60)	12
Unknown	Plasma	Case Report, Confirmed Glucagonoma (Female)	1	271 (Cited Normal Range 40 ~ 140)	13
RIA	Serum	10 Female + 9 Male (Bioreclamation Donors)	19	65.1 ~ 189.4	14

Table 1: **Glucagon levels in various states.** Highlighted glucagon levels represent the most severe pathophysiological states.

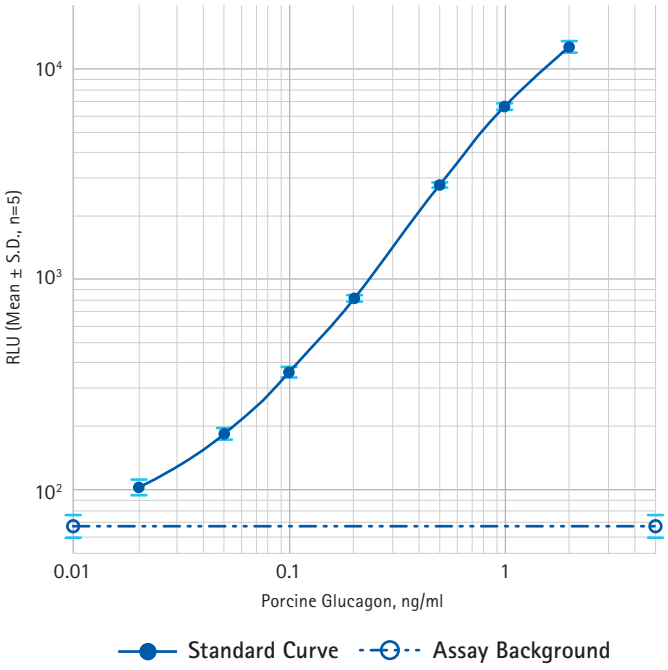

Figure 1. **EMD Millipore's Glucagon ELISA (Cat. No. EZGLU-30K) kit exhibits sensitive, precise detection of full length, porcine glucagon with low inter-assay variation.** The standard curve was generated using purified World Health Organization porcine glucagon international standard.

The development of an effective glucagon ELISA has been long delayed because of the difficulty in raising specific antibodies to mature glucagon. Mature glucagon is a peptide of 29 amino acids and its amino acid sequence is highly conserved across the animal kingdom. Glucagon is synthesized from preproglucagon gene in pancreatic α-cells, processed and then secreted into bloodstream. However, many immunoreactive glucagon forms have been identified in blood, including:

1. big plasma glucagon (~ 60,000 Da)
2. proglucagon (~ 9,000 Da)
3. authentic glucagon
4. small glucagons (~ 2,000 Da)

In addition, the translational product from the same preproglucagon gene in intestine is processed into oxyntomodulin (OXM), which is glucagon with an 8-amino acid extension at C-terminal end[15]. OXM is also released into circulation and further increases the difficulty of developing a glucagon-specific immunoassay.

Here, we describe the application of a new, highly specific glucagon chemiluminescent ELISA kit with no cross-reactivity to miniglucagon or glucagon 1–18 and < 5% cross-reactivity to OXM (data not shown). A representative standard curve is shown in Figure 1.

Another challenge of measuring glucagon in blood samples is interference from serum or plasma matrix components. Therefore, samples need to be extracted prior to glucagon immunoassay. Solid phase extraction (SPE) is a common method of removing matrix components from blood samples; however, this method can be costly. We present a rapid, inexpensive method for extracting serum or plasma samples that enriches the sample for glucagon at a fraction of the cost of solid phase extraction.

Materials and Methods

Sample preparation, rapid procedure. Plasma and serum samples were collected with 500 kU/mL aprotinin and extracted using our rapid extraction method to eliminate blood matrix interference. Briefly, acetonitrile (ACN) was added to a final concentration of 60% in each sample. For human and rat samples, for example, 300 µL sample was mixed with 450 µL acetonitrile. Samples were vortexed immediately and centrifuged at 17,000 x g for 5 minutes. Supernatants were removed and samples were dried. Dried samples were rehydrated in 60 µL assay buffer to achieve 4-fold concentration over initial concentration. 20 µL was used for each assay, in duplicate.

Comparison of sample preparation using rapid procedure and using solid phase extraction plate. 21 paired human plasma samples were extracted using the sample preparation, rapid procedure (above) and the Waters Oasis® HLB Plate. This 96-well plate contains the Oasis® HLB sorbent, which is a universal polymeric reversed-phase sorbent that was developed for the extraction of a wide range of acidic, basic, and neutral compounds from various matrices. Extraction was conducted according to the manufacturer's recommended protocol. Both sets of samples were dried and reconstituted in assay buffer. 20 µL was used for measurement of glucagon by ELISA using the chemiluminescent substrate.

Assessing recovery of spiked analyte following extraction. 0.25 mL human plasma samples were spiked with 5 µL of human glucagon to achieve final concentrations of either 44 pg/mL or 248.5 pg/mL glucagon. Samples were mixed and extracted with 60% ACN using the rapid procedure as above. Dried extracts were reconstituted in assay buffer equivalent to ¼ of original plasma volume. Glucagon was measured using the EMD Millipore Glucagon ELISA Kit (Cat. No. EZGLU-30K).

Assessing assay linearity by sample dilution. 5 µL human glucagon (100 ng/mL) was spiked into 250 µL human plasma and extracted with 60% ACN. 0.55 mL of supernatant from each extraction was dried and reconstituted in 0.25 mL assay buffer. Various sample volumes (5, 10, 15 and 20 µL) were used for assay using the EMD Millipore Glucagon ELISA (Cat. No. EZGLU-30K).

Evaluation of multiple extractions of the same sample. Pooled human serum or plasma (n = 12 each) were spiked with glucagon to various levels and five 250 µL aliquots were prepared for parallel extractions by 60% ACN. Each extraction was performed independently. We also obtained plasma samples from 8 human plasma donors and performed two extractions in parallel from each sample. Glucagon levels were measured for each extraction and compared.

Assay procedure. Wells were rinsed 3 times with wash buffer. 10 µL assay buffer, 20 µL glucagon standard or sample and 20 µL of the antibody mixture were mixed in each well and incubated at room temperature (RT) for 3 hours on a microplate shaker. The assay mixture was decanted and wells rinsed 3x with wash buffer. 100 µL streptavidin-horseradish peroxidase enzyme solution was added to each well and incubated for 30 min at RT with shaking. The enzyme solution was removed, wells rinsed 6x with wash buffer, and chemiluminescent substrate added. Within 5 min of substrate addition, light intensity was measured in a luminometer at 425 nm. Another small volume procedure with longer incubation time is also available. (see product package insert for details).

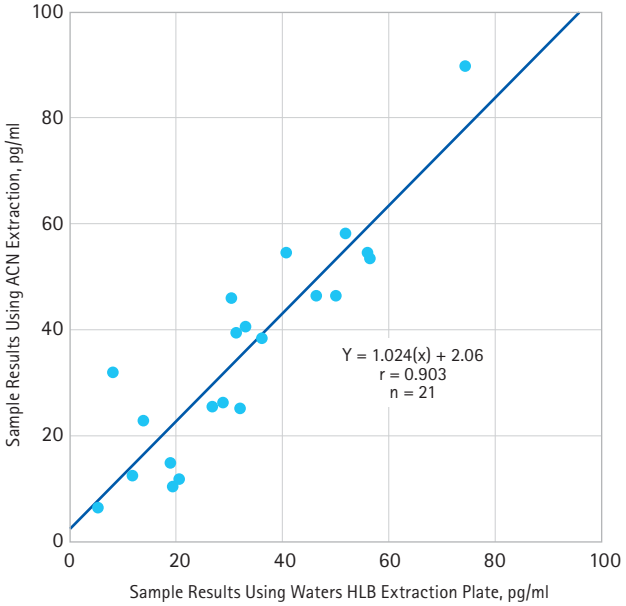

Figure 2. Correlation of glucagon measurements following sample extraction using rapid acetonitrile extraction (y-axis) with measurements following extraction using solid phase extraction (x-axis).

Results

To eliminate adverse matrix effects on glucagon quantification, the interference factors in serum/plasma samples have to be removed by extraction. Although solid phase extraction is effective, it can be costly (over $300 per 96-well plate). We developed a simple and inexpensive acetonitrile extraction method which yielded glucagon measurements that showed good correlation (r = 0.903) with measurements obtained following solid phase extraction (Figure 2). This simple acetonitrile extraction method is not only inexpensive, it is also fast; with an appropriate drying apparatus such as a SpeedVac® Concentrator, the entire extraction can be done within 4 or 5 hours.

For accurate ELISA quantification, sample extraction methods need to exhibit high recovery, ensuring that the amount of analyte measured reflects the level of analyte in the subject's bloodstream. We tested the recovery rate of our rapid acetonitrile extraction method by spiking plasma samples with low and high levels of purified glucagon. After extraction, samples were analyzed using the EMD Millipore Glucagon ELISA kit (Cat. No. EZGLU-30K). For low level spiked samples, we observed a mean recovery rate of 93.0 +/- 2.83%, and for high level spiked samples, we observed 83.7 +/- 1.64%.

	Glucagon Level				
	Basal	Spiked at Low Level		Spiked at High Level	
Sample #	pg/mL	pg/mL	Recovery	pg/mL	Recovery
1	15.8	55.8	90.1%	226.8	84.9%
2	11.5	51.5	90.1%	217.3	82.8%
3	25.5	67.8	96.0%	239.0	85.9%
4	59.0	101.0	95.5%	262.5	81.9%
5	50.0	91.0	93.2%	256.3	83.0%
Mean ± S.D. (n = 5)	—	—	**93.0 ± 2.83%**	—	**83.7 ± 1.64%**

Table 2. **High recovery of spiked glucagon in plasma samples following rapid acetonitrile extraction.** Five plasma samples were spiked with low levels (44 pg/mL) or high levels (248.5 pg/mL) of glucagon, extracted and analyzed using the EMD Millipore Glucagon ELISA kit (Cat. No. EZGLU-30K).

To assess the linearity of the glucagon ELISA kit, we spiked human plasma sample replicates with a known amount of glucagon, extracted the samples, and measured glucagon levels from various amounts of resuspended sample (Table 3). In all cases, the amount of glucagon observed was 100% of the amount expected.

	Glucagon Content Measured at Various Sample Volumes							
	20 µL		15 µL		10 µL		5 µL	
Sample #	ng/mL	% of Expected	ng/mL	% of Expected	ng/mL	% of Expected	ng/mL	% of Expected
1	1.903	100	1.433	100.4	0.942	99.0	0.472	99.1
2	1.876	100	1.410	100.2	0.942	100.4	0.473	100.8
3	1.914	100	1.399	97.5	0.957	100.0	0.469	97.9
4	1.840	100	1.440	104.3	0.948	103.0	0.492	107.0
Mean ± S.D.		100	——	100.6 ± 2.80	——	100.6 ± 1.70	——	101.2 ± 4.05

Table 3. **Linearity of glucagon ELISA response to varying amounts of sample.** Varying amounts of glucagon-spiked samples were extracted and measured using the EMD Millipore Glucagon ELISA Kit (Cat. No. EZGLU-30K).

Next, we tested the reproducibility of the extraction method via two separate experiments. First, we conducted five extractions in parallel for each of 4 replicate samples of serum and of plasma, both spiked with glucagon. Table 4 shows the mean glucagon levels observed as well as the coefficient of variation for each sample, showing that, overall, the rapid acetonitrile extraction method is reproducible. Second, we performed parallel, duplicate extractions for 8 samples from human plasma donors, and measured the amount of glucagon in each independently extracted replicate. The results (Figure 3) show nearly identical amounts of glucagon measured for each set of duplicate extractions, also indicating that the extraction method was highly reproducible.

		Measured Glucagon (pg/mL) in Extraction Runs						
Sample Type	Sample #	1st	2nd	3rd	4th	5th	Mean ± S.D.	C.V.
Serum	1	31.5	32.0	32.3	33.0	33.8	32.5 ± 0.88	2.72 %
	2	103.3	102.0	111.5	113.0	109.5	107.9 ± 4.95	4.59 %
	3	180.8	180.3	184.3	175.8	186.3	181.5 ± 4.04	2.23 %
	4	318.3	337.5	338.0	333.0	326.0	330.6 ± 8.39	2.54 %
Plasma	1	14.3	12.8	15.0	20.0	13.3	15.1 ± 2.90	19.30 %
	2	88.0	85.0	84.3	89.0	88.8	86.9 ± 2.09	2.40 %
	3	176.0	163.3	173.8	181.8	163.8	171.7 ± 8.04	4.68 %
	4	323.8	297.3	306.0	324.3	305.8	311.4 ± 12.0	3.86 %

Table 4. **Evaluation of multiple extractions of the same sample.**

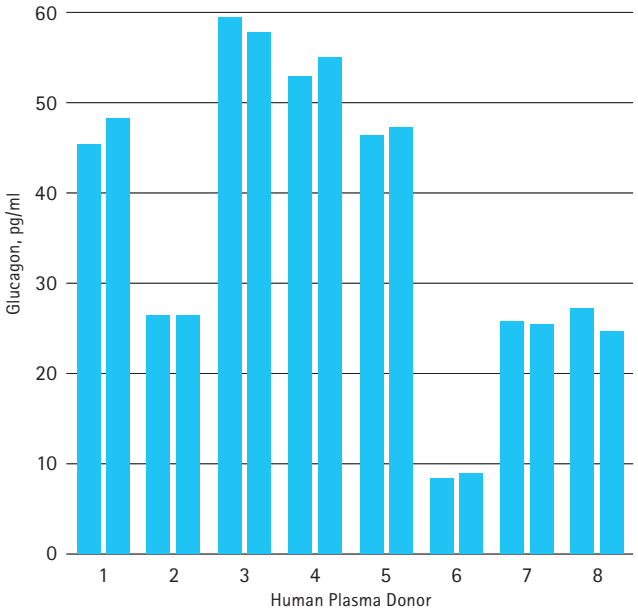

Figure 3. **Replications of sample extraction.** Nearly identical glucagon levels observed following duplicate extractions of human plasma samples showed reproducibility of the extraction method.

Finally, we tested the relative accuracy of the EMD Millipore Glucagon ELISA Kit (Cat. No. EZGLU-30K) by measuring glucagon levels in 34 samples using both the EMD Millipore kit and a glucagon ELISA from supplier A. We also measured glucagon in another set of 16 samples using ELISA kits from EMD Millipore and supplier B. Unlike the glucagon levels detected using ELISA kits from suppliers A and B, ELISA results of serum or plasma glucagon levels in normal humans were very comparable to what had been reported in the literature (compare Table 5 and 6 to Table 1). Neither competitor kit states a requirement for sample extraction; however, using competitor B's kit, most samples were below detection limits (some were highly false positive) and using competitor A's kit, 20% of the samples from normal subjects exhibited glucagon levels higher than 200 pg/mL (higher than normal range reported in the literature—Table 1). Findings indicate that EMD Millipore's Glucagon ELISA Kit (Cat. No. EZGLU-30K) is more accurate than kits of two competing suppliers.

Sample #	EMD Millipore	Competitor A	Sample #	EMD Millipore	Competitor A
1	26.30	73	18	22.50	130
2	46.40	80	19	41.50	76
3	27.30	267	20	67.30	63
4	58.10	140	21	30.30	153
5	53.30	156	22	44.50	88
6	53.50	101	23	16.00	114
7	7.50	249	24	41.50	69
8	46.40	215	25	18.50	65
9	13.00	74	26	9.00	79
10	8.25	51	27	16.30	242
11	22.00	73	28	84.00	224
12	25.10	58	29	11.30	100
13	21.00	79	30	13.50	84
14	36.50	62	31	21.30	197
15	18.00	267	32	19.80	171
16	35.50	186	33	44.50	23
17	13.00	218	34	45.80	20

Table 5. **Comparison of glucagon levels (pg/mL) measured in human plasma samples using ELISA kits from EMD Millipore and Competitor A.**

Sample #	EMD Millipore	Competitor B	Sample #	EMD Millipore	Competitor B
1	7.5	3,629	9	30.3	—
2	46.4	3,460	10	44.5	1,060
3	22.0	—	11	18.5	—
4	25.1	383	12	9.0	—
5	21.0	—	13	11.3	—
6	36.5	305	14	13.5	—
7	13.0	—	15	21.3	—
8	22.5	—	16	19.8	—

Table 6. **Comparison of glucagon levels (pg/mL) measured in human plasma samples using ELISA kits from EMD Millipore and Competitor B. (— = Undetectable)**

Conclusion

To address the need for specifically detecting full length glucagon without confounding signals from other glucagon-like polypeptides in complex samples, we have developed a glucagon ELISA kit that may be used to accelerate studies of this therapeutically important hormone in normal and disease states.

Starting with 300 µL serum/plasma sample, this assay, including sample extraction, can be completed within a working day. With slight modifications and longer incubation time (24 ~ 48 hours), the same kit can be used to measure glucagon in mouse serum/plasma. The rapid method of sample extraction presented here is robust, and leads to reliable assay results.

The level of glucagon in plasma samples from normal human subjects as measured using the EMD Millipore ELISA kit, in contrast to ELISA kits from competing suppliers, agree well with published glucagon levels derived from RIA, the long-held gold standard method for glucagon assay. Therefore, this ELISA kit should be easily incorporated into new and ongoing research, preclinical and clinical studies of metabolism.

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Ordering Information and Related Products

ELISA Kits

Description	Species	Qty.	Catalogue No.
Glucagon	Multi-species	1	EZGLU-30K
		10	EZGLU-30BK
GLP-1	Multi-species	1	EGLP-35K
		10	EGLP-35BK
GLP-2	Human/Rat	1	EZGLP2-37K
Insulin	Human	1	EZHI-14K
		10	EZHI-14BK

RIA Kits

Description	Species	Catalogue No.
Glucagon	Multi-species	GL-32K
Insulin	Rat	RI-13K
Insulin	Human	HI-14K

MILLIPLEX® MAP Kits

Description	Catalogue No.
Human Metabolic Hormone Magnetic Bead Panel	HMHMAG-34K
Metabolic Magnetic Bead Magnetic Bead Panel	MMHMAG-44K
Rat Metabolic Magnetic Magnetic Bead Panel	RMHMAG-84K

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