

WHITE PAPER

GLUCAGON MEASUREMENT

Addressing Long-Standing Analytical Challenges

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LOOK INSIDE FOR ANSWERS TO THESE QUESTIONS:

- What technical aspects of commercial glucagon assays have prevented them from meeting scientists' needs?
- Are there analytical solutions available to meet those needs?

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Glucagon is a fundamental regulator of glycemic control, through its ability to stimulate hepatic glucose production, and numerous studies have demonstrated that there is dysregulation of glucagon in all forms of diabetes. Interest in this pancreatic alpha cell hormone has grown considerably in recent years and with advances in the drug development arena, the need to address long-standing analytical obstacles has become even more urgent.

Mercodia has developed two novel glucagon assays that address the technical issues that have plagued analytical measurement of this biomarker for many years. The Mercodia Glucagon ELISA (10-1271-01) was developed for human samples and the Mercodia Glucagon ELISA – 10uL (10-1281-01) was developed for use with animal samples. The long-standing technical barriers and the ways in which Mercodia’s two new assays address these challenges are detailed in the following sections.

Specificity Challenges Lead to a Terminal End, Dual Antibody Approach

Glucagon is a peptide hormone that is processed from a much larger precursor, proglucagon. The pre-proglucagon gene is expressed in specific portions of the brain (i.e., medulla oblongata), intestines (i.e., L cells) and pancreas (i.e., alpha cells) (1). Thus, the resulting 160 amino acid prohormone proglucagon is produced by several different cell types; however, the primary ways in which it is posttranslationally processed to biologically active peptides have to do with “location, location, location”. The expression and activity of prohormone convertase 2 (PC2/PCSK2) in pancreatic alpha cells gives rise to the 29 amino acid polypeptide glucagon, among other, lesser-understood proteins (Figure 1). It is interesting to note that glucagon can be partially degraded by an endopeptidase, resulting in a truncated form known as glucagon 19-29 or miniglucagon. Miniglucagon is present in islets and glucagon-target tissues at very low concentrations but appears to be a highly potent regulator of physiological processes in these tissues. It has also been found to have opposite actions than that of its full-length parent peptide, glucagon (2, 3, 4).

Cleavage by PC2 is not the only way in which proglucagon is processed. The proconvertase enzyme PC1/3 (PCSK1/3), expressed by the intestinal L cells and in the brain, recognizes different regions of proglucagon and results in the production of a number of proteins, including glicentin, oxyntomodulin, GLP-1 and GLP-2 (Figure 1).

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“Circulating levels of homologous proteins should also be taken into consideration and can make cross-reactivity in a glucagon assay particularly problematic, which is especially true for glicentin.”

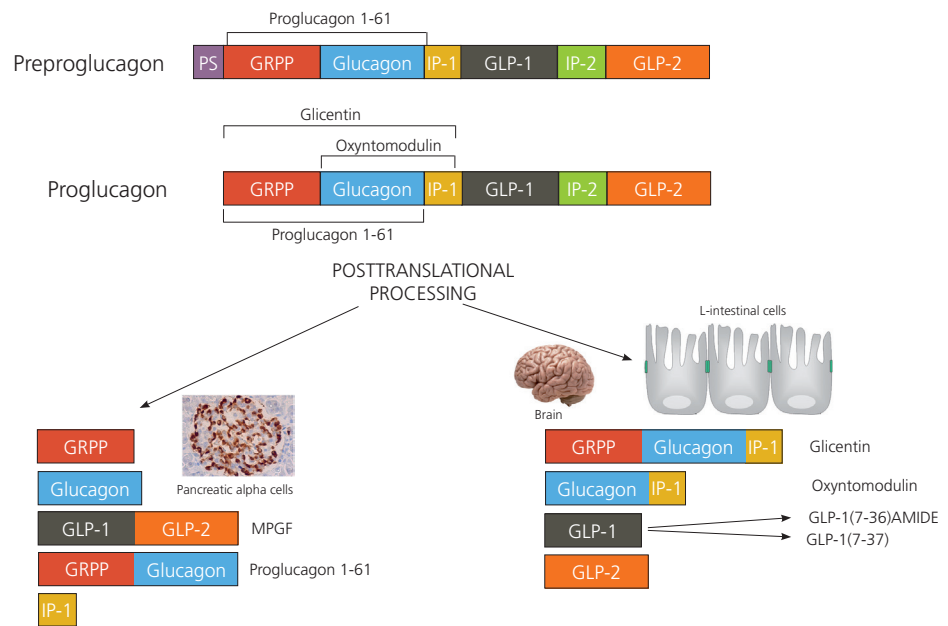


Figure 1. Posttranslational Processing of Proglucagon

While these are the predominant ways in which proglucagon is processed in a tissue-specific manner, reports of hyperglucagonemia and glucagon reactivity after total pancreatectomy have opened up the possibility that PC2 may also be expressed in the gut under certain conditions (5). Furthermore, GLP-1 production by alpha cells has been demonstrated in animal models and isolated human islets (6, 7, 8).

Glucagon	HS QGTFTSDYSKYLDSSR AQDFVQWLMNT
Oxyntomodulin	HS QGTFTSDYSKYLDSSR AQDFVQWLMNTKRNRNNIA
Glicentin	RSLQDTEEKSRFSASQADPLSDPDQMNEKDR HSQGTFTSDYSKYLDSSR AQDFVQWLMNTKRNRNNIA

Figure 2. Glucagon, Oxyntomodulin and Glicentin Amino Acid Sequences

Posttranslational processing of proglucagon is important to consider when measuring glucagon because the end products are not all distinctly different proteins with completely unique sequences. While glucagon's actions may be unique, its sequence is not. Herein lies a major part of the measurement quandary that has faced scientists in years past.

A number of proteins contain glucagon's full sequence, including the 61 amino acid processing product proglucagon 1-61. The full-length glucagon amino acid sequence also makes up a large portion of glicentin and oxyntomodulin (Figure 2). For this reason, these two gut hormones have been called "enteroglucagon" or "gut glucagon" (9). This sequence homology makes measuring glucagon particularly difficult since antibodies used in glucagon assays may recognize epitopes shared by all of the aforementioned hormones.

Circulating levels of homologous proteins should also be taken into consideration and can make cross-reactivity in a glucagon assay particularly problematic, which is especially true for glicentin. Proglucagon 1-61 is said to represent only a negligible fraction of immunoreactive glucagon, although samples from pancreatectomized patients or those in renal failure may contain elevated levels (10).

"Inaccurate glucagon readings can have enormous negative implications on research and clinical decisions."

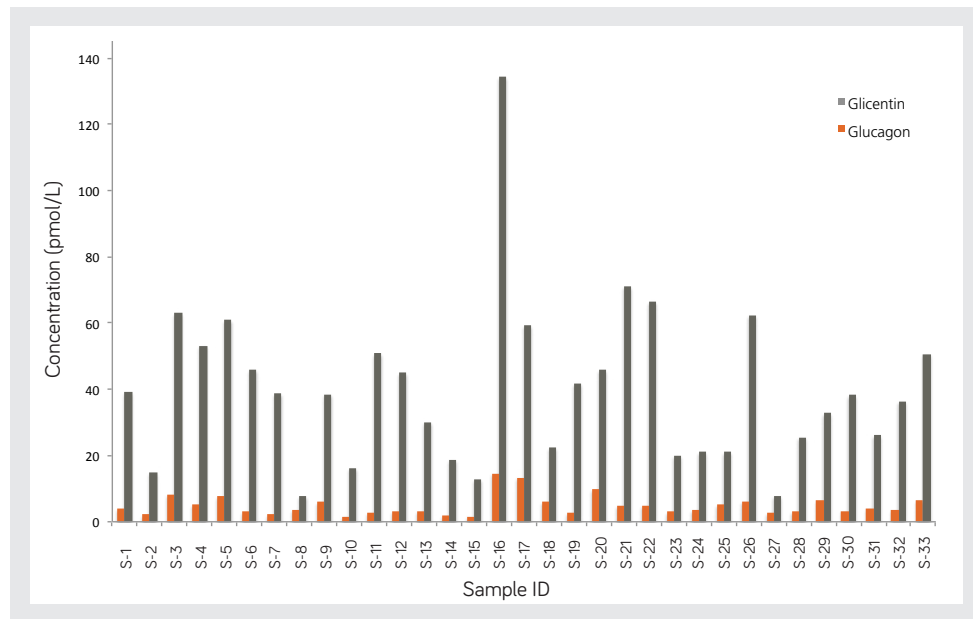


Figure 3. Glucagon and Glicentin Concentrations in Human Plasma Samples ($R^2 = 0.52$, $n = 33$), detected using the Mercodia Glucagon ELISA (10-1271-01) and Mercodia Glicentin ELISA (10-1273-01), respectively

In the case of oxyntomodulin, the current understanding is that it circulates at detectable but low levels (11), however further work is needed due to the lack of reliable oxyntomodulin assays. In a study published in 2014, commercially available assays for oxyntomodulin were compared. After an extensive evaluation of those assays, the authors concluded "that none of the assays carried out with the tested commercially available kits can measure human oxyntomodulin (or glicentin) concentrations with any reliability" (12).

While these other proteins appear to be present at low levels in circulation, the same is not true for glicentin. Glicentin circulates at much higher levels than glucagon, as demonstrated by the use of new glucagon- and glicentin-specific assays (Figure 3). The issue of cross-reactivity is further confounded by the fact that these hormones are regulated differently, which means that a "blanket" or background subtraction may not be able to be utilized to correct values from a glucagon assay that has cross-reactivity to glicentin. These issues raise questions about true glucagon concentrations reported by assays that cross-react with glicentin or do not report cross-reactivity to relevant homologous proteins. Inaccurate glucagon readings can have enormous negative implications on research and clinical decisions.

The use of an assay that cross-reacts with other glucagon-containing proteins may result in inaccurate characterization of alpha cell function and glucagon kinetics under various physio-

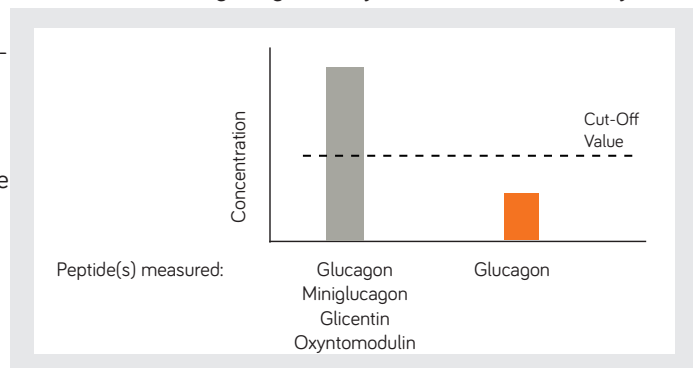


Figure 4. Glucagon Assay Results Based on Specificity Differences

"A true insulin:glucagon ratio cannot be determined if using assays (for either biomarker) that are not highly specific."

logical conditions, as well as misinterpretation of how a subject is responding to therapy. Falsely elevated levels may also negatively impact decisions on inclusion of subjects in studies or stratifications based on specific glucagon cut-off values (Figure 4).

Dynamic changes in both insulin and glucagon are fundamental to the progression of diabetes and thus, determination of the insulin:glucagon ratio has been used for many years in research as a more comprehensive metabolic index than measuring insulin alone (13, 14, 15, 16, 17). This ratio is not only important to the study of diabetes but provides critical insight into the storage and utilization of nutrients in normal as well as catabolic states such as infection, trauma, cancer, etc. (13). A true insulin:glucagon ratio cannot be determined if using assays (for either biomarker) that are not highly specific. While proglucagon 1-61, oxyntomodulin and glicentin contain the full glucagon sequence, they have extensions at the N-terminal and/or C-terminal ends (Figures 1 and 2).

Therefore, to address the need for a highly specific glucagon assay, Mercodia took a dual antibody approach, targeting both terminal ends of glucagon. This strategy was also chosen in light of the fact that truncated forms of glucagon have been reported. These shorter forms include previously mentioned miniglucagon, as well as glucagon 3-29 and 5-29, which are produced by sequential degradation (and inactivation) by dipeptidyl peptidase IV (DPPIV) (18, 19).

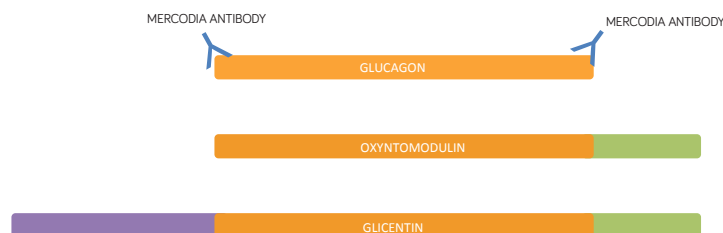


Figure 5. Positional Binding of Monoclonal Antibodies in Mercodia Glucagon ELISAs

Mercodia developed two highly specific mouse monoclonal antibodies, characterizing them extensively using ELISA and Biacore techniques. These two antibodies are used in both of the Mercodia glucagon assays (Figure 5) and cross-reactivity testing revealed excellent specificity profiles (Figure 6). Initial specificity testing included proglucagon-derived proteins that are more commonly studied and more prominent in circulation. Subsequent studies were conducted to examine the cross-reactivity of proglucagon 1-61 and glucagon 3-29 in the Mercodia Glucagon ELISA (10-1271-01), as more is known about these peptides in human samples. Biacore analysis demonstrated that the capture antibody binds full-length glucagon 70x stronger than proglucagon 1-61.

The Mercodia Glucagon ELISA (10-1271-01) has 5-10% cross-reactivity to proglucagon 1-61 when using 10-200 pmol/L of the peptide. As previously mentioned, proglucagon 1-61 normally circulates at low levels but may be increased under specific conditions. The low cross-reactivity of proglucagon 1-61 in the Mercodia Glucagon ELISA can be further reduced by using an alternative format (contact Mercodia for details). Biacore and ELISA analyses showed that there is no cross-reactivity to glucagon 3-29 at or above physiological concentrations. This is also an important finding because glucagon 3-29 has been reported to be the major metabolite in clinical samples due, in large part, to *in vitro* plasma protease metabolism during sample storage (20).

“This increased specificity is a primary reason why concentrations in the Mercodia glucagon assays are lower than concentrations generated by other glucagon assays.”

Mercodia Glucagon ELISA (10-1271-01)	Cross-Reactivity	Mercodia Glucagon ELISA 10 µL (10-1281-01)	Cross-Reactivity
Miniglucagon (Glucagon 19-29)	< 0.1%	Miniglucagon (Glucagon 19-29)	n.d.
Glicentin	0.8%	Glicentin, human	1.0%
Oxyntomodulin	4.4%	Glicentin, mouse	7.0%
GLP-1 (1-36 amide)	< 0.3%	Glicentin, rat	4.0%
GLP-1 (9-36 amide)	< 0.3%	Oxyntomodulin human/rat/mouse	2.0%
GLP-1 (1-37)	< 0.3%	Oxyntomodulin bovine/canine/porcine	n.d.
GLP-2	< 0.3%	GLP-1 (7-36)	n.d.
GRRP	< 0.0005%	GLP-1 (9-36)	n.d.
		GLP-2	n.d.

Figure 6. Specificity Charts for the Mercodia Glucagon ELISAs

Mercodia's careful selection of glucagon antibody clones ensured that a true sandwich formation would only be possible in the presence of glucagon, helping to prevent cross-reactivity to related proteins that contain the full (e.g., glicentin) or partial (e.g., miniglucagon) glucagon sequence. This increased specificity is a primary reason why concentrations in the Mercodia glucagon assays are lower than concentrations generated by other glucagon assays (Figure 4).

Sensitivity Limitations Finally Addressed

It is not only important to study glucagon levels as they rise and peak but it is equally important to be able to detect glucagon levels as they decline. One must use an assay that is highly specific so changes are not masked by the levels of cross-reacting proteins but the assay must also be able to accurately quantify low levels of glucagon so such changes can be reliably measured and monitored over time.

Glucagon circulates at low concentrations, around 10 pmol/L (35 pg/mL) or less and can increase to ~20-30 pmol/L (70 – 105 pg/mL) during hypoglycemic conditions and can drop down to 1-2 pmol/L (3.48 – 7 pg/mL) during hyperglycemia (21, 22). Even though the physiological range of glucagon is reported to be 0 – 30 pmol/L (0 – 105 pg/mL), for many years, commercially available glucagon assays did not provide sufficient sensitivity for detecting glucagon under a variety of physiological conditions. In a comparison of multiple commercially available glucagon assays, the assay found to be most sensitive had a sensitivity of approximately 10 pmol/L (35 pg/mL) but results around this concentration were not reliable. The authors concluded that both the sensitivity and precision of the assays tested were generally poor. They state: “Clearly, assays with sensitivities >5 pmol/l are, therefore, unsuitable for the complete characterization of glucagon secretion.” In addition, some assays had poor recovery in plasma and/or buffer, the assays reported variable baseline concentrations and glucagon was not always detected, particularly under conditions in which it was suppressed (12).

In order to provide the scientific community with the ability to detect glucagon under various physiological conditions, a novel glucagon assay must offer an advantage over the measuring range of insensitive assays on the market. The Mercodia glucagon assays for human and animal samples have sensitivities of 1.5 pmol/L (5 pg/mL) and 2 pmol/L (7 pg/mL), respectively. These novel methods offer significant improvements over what was previously available (i.e., sensitivities of 10pmol/L or greater) and meet the needs of researchers as discussed by Bak et al, who state that test methods with sensitivities >5 pmol/L (17 pg/mL) do not allow for full characterization of glucagon (12).

The sensitivities of Mercodia's two assays, coupled with broad dynamic ranges, afford scientists the ability to measure physiologically relevant concentrations of glucagon in a variety of experimental paradigms.

Overcoming Sample Volume Restrictions

Sample volume requirements have also significantly limited the use of glucagon assays. Most commercially available methods require at least 50-100uL of plasma (12). This has direct implications on the number of analytes that can be measured, the number of time points that can be examined and thus, the scope of scientists' conclusions.

The Mercodia glucagon ELISAs were developed and optimized to require minimal sample volumes, with the assay for human samples requiring only 25uL and the assay for animal samples requiring only 10uL of sample. These very low sample volumes offer a significant advantage over requirements of traditional assays and enable scientists to measure glucagon along with other relevant hormones and/or study temporal changes in glucagon in a variety of experimental models.

Human	H S Q G T F T S D Y S K Y L D S R R A Q D F V Q W L M N T
Rhesus	- - - - -
Mouse	- - - - -
Rat	- - - - -
Dog	- - - - -
Cat	- - - - -
Porcine	- - - - -
Bovine	- - - - -
Chicken	- - - - - S -

Figure 7. Glucagon Sequence Across Species

“Clearly, assays with sensitivities >5 pmol/l are, therefore, unsuitable for the complete characterization of glucagon secretion.”
(Bak et al. 2014)

“The 10µL Mercodia Glucagon ELISA has been validated with mouse, rat, pig and non-human primate samples...”

Implications for Translational Research

A “bench-to-bedside” approach is important to the understanding of physiological and pathophysiological processes, as well as the development of therapeutic strategies. Mercodia’s two glucagon assays provide analytical solutions for studies that span the spectrum of translational research based on extensive work that was conducted to characterize how these methods perform with different sample types.

Glucagon’s sequence is highly conserved across species (Figure 7) but validation of different sample types is crucial because sequence homology is not the only factor that affects antibody-antigen binding in an immunoassay. For example, matrix interferences (common in animal samples) can lead to falsely elevated or falsely low concentrations. Mercodia assays contain a unique blocking solution to prevent or minimize matrix interferences, especially important for the 10uL assay, which has been optimized for animal samples.

The 10uL Mercodia Glucagon ELISA has been validated with mouse, rat, pig and non-human primate samples (data available in the Directions For Use), making it an excellent choice for a variety of small and large animal preclinical studies. Both Mercodia glucagon assays can be used with samples from cultured cells, providing accurate glucagon measurement options for those conducting *in vitro* research (e.g., conversion of alpha to beta cells or vice versa, human embryonic stem cells to alpha or beta cells, production of islet-like clusters).

The Mercodia glucagon assays are based on the same highly specific monoclonal antibodies and cross-comparisons have been conducted (internal data), making them excellent choices for translational studies especially since measurement reliability and continuity across phases can be critically important.

“the Mercodia assay has the best performance, in terms of specificity, precision and sensitivity data.”

(Wewer Albrechtsen *et al.* 2014)

Independent Evaluation and Assay Comparison

The Mercodia Glucagon ELISA (10-1271-01) was included in an independent comparison study led by Dr. Jens Juul Holst. This was a follow-up to their previously mentioned assay comparison work published in early 2014, in which they demonstrated that key performance characteristics of commercially available glucagon assays were significantly lacking (12).

Mercodia’s assay was included in their subsequent study of three newly available tests. Based on their extensive evaluation and expertise in this field, they determined that “the Mercodia assay has the best performance, in terms of specificity, precision and sensitivity data”. They demonstrated that the Mercodia ELISA yielded reproducible results between runs performed with different lots and full recovery of glucagon in both buffer and plasma. Based on their findings, they concluded that only the Mercodia assay was “suitable for measuring glucagon concentrations in clinical samples”(23).

...they concluded that only the Mercodia assay was “suitable for measuring glucagon concentrations in clinical samples”.

(Wewer Albrechtsen *et al.* 2014)

Conclusion

Measuring glucagon has been difficult in the past because assays lacked performance characteristics that meet the needs of the scientific community.

Mercodia’s two novel glucagon assays provide high quality solutions by offering excellent specificity, optimal sensitivity and low sample volume requirements. These assays are easy-to-use, requiring no laborious and time-consuming purifications or pre-treatments, and do not involve the use of radioactivity. Furthermore, they can be used in basic, preclinical and clinical studies.

Mercodia’s new glucagon ELISAs are valuable analytical tools that enable the accurate and reliable measurement of this important metabolic biomarker in translational studies.

For research-based presentations on glucagon, by leading experts, visit
www.mercodia.com/webinars.

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