

WHITE PAPER

# How the introduction of a specific glicentin ELISA made glucagon measurements even more specific

## Know what you measure

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## Measuring glucagon – not so simple!

Glucagon, a fundamental regulator of glycemic control, acts via its ability to stimulate hepatic glucose production: numerous studies have demonstrated its dysregulation in all forms of diabetes. It comprises a peptide hormone processed from the much larger precursor protein proglucagon, whose encoding gene (GCG) has been found to be expressed by pancreatic  $\alpha$ -cells, L-intestinal cells in the gut, and the brain stem<sup>1</sup>. The resulting 160 amino acid prohormone proglucagon is thus produced by several different cell types and, depending on what processing enzymes are present in the expressing cell, its post-translational processing gives rise to different protein products.

Figure 1 shows an overview of proglucagon processing in different tissues as regulated by differential expression of specific prohormone convertases<sup>1</sup>.

*“Post-translational processing of proglucagon is important to consider when measuring glucagon.”*

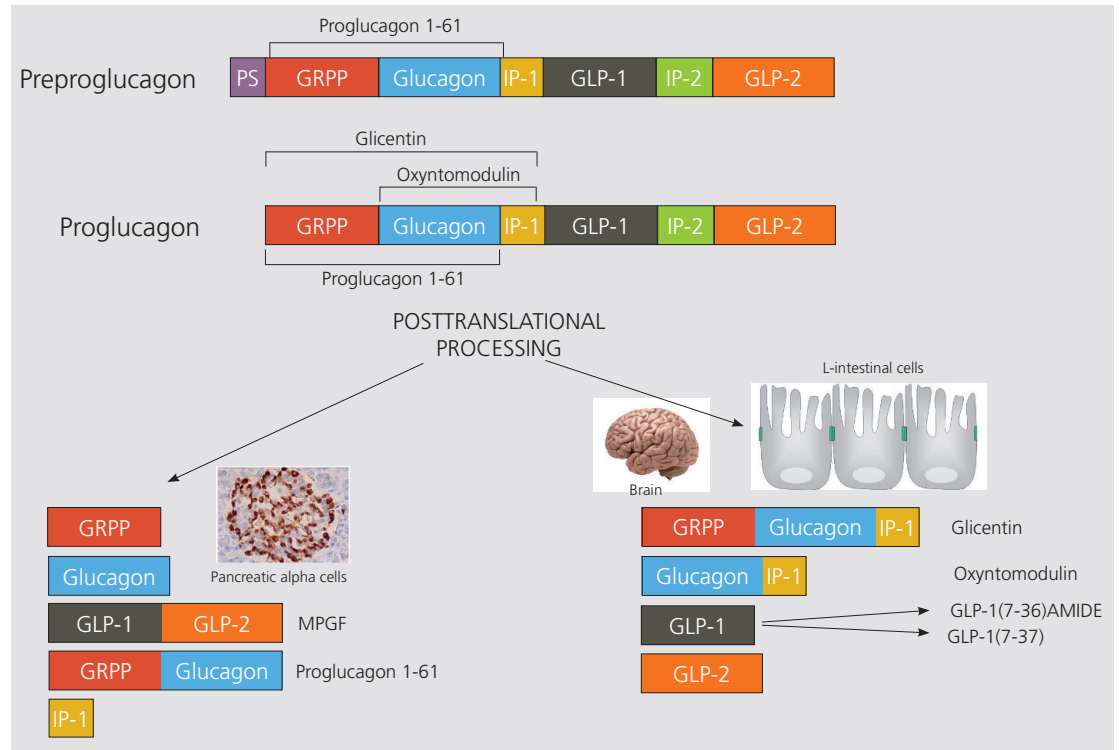


Figure 1. Post-translational processing of proglucagon results in several different protein.

Post-translational 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 measurement dilemma that scientists have faced in years past.

Even though glucagon is considered to be largely produced and secreted by pancreatic  $\alpha$ -cells, it was nevertheless suggested in 2016 that the gut’s L-cells can also secrete glucagon since it was found in the circulation in pancreatectomized patients<sup>2</sup>. Also, low amounts of GLP-1 have been shown to be secreted by pancreatic islets and seem to be increased in diabetes<sup>3</sup>.

Glicentin, oxyntomodulin and proglucagon 1-61, all proteins derived from proglucagon, contain the full amino acid residue sequence of glucagon. Glicentin and oxyntomodulin are mainly produced and secreted from L-cells, while proglucagon 1-61 has been shown to be produced by  $\alpha$ -cells in the pancreatic islet<sup>1,4</sup>. Recent studies have shown that under certain metabolic conditions, the processing of proglucagon can be significantly altered.

*“Recent studies have shown that under certain metabolic conditions, the processing of proglucagon can be significantly altered.”*

## Development of a specific glucagon ELISA

To address the need for a highly-specific glucagon assay, Merck developed two glucagon ELISAs (art no 10-1271-01 and 10-1281-01) launched in 2013 and 2014, respectively, each with a dual-antibody approach targeting both terminal ends of the glucagon peptide. This strategy was also chosen in light of the fact that truncated forms of glucagon have been reported. These shorter forms include mini-glucagon, as well as glucagon 3-29 and 5-29.



Figure 2. Positional binding of monoclonal antibodies in Merck Glucagon ELISAs.

Merck developed two highly-specific mouse monoclonal antibodies that were characterized extensively using ELISA and Biacore techniques. These two antibodies are used in both Merck glucagon assays (Figure 2) where cross-reactivity testing revealed excellent specificity profiles (Table 1). Initial specificity testing included proglucagon-derived proteins that are prominent in circulation. Subsequent studies were conducted to examine the cross-reactivity of proglucagon 1-61 and glucagon 3-29 in Merck Glucagon ELISA (10-1271-01) since more is known about these peptides in human samples. Biacore analysis demonstrated that the capture antibody binds full-length glucagon 70-fold stronger than proglucagon 1-61.

Table 1. Specificity data for Merck Glucagon ELISAs.

<b>Specificity</b>		
the following crossreactions have been found:		
	<b>Crossreaction (%)</b>	<b>Highest concentrations tested (pmol/L)</b>
Glicentin	0.8	300
Oxyntomodulin	4.4	135
Mini-glucagon	<0.1	1600
GLP-1	<0.3	500
GLP-2	<0.3	500
GRPP	<0.0005	300000
GlucaGen (Recombinant glucagon for injections)	100	100

Merck's careful selection of glucagon antibody clones resulted in a true sandwich formation that would only be possible in the presence of glucagon, thus preventing cross-reactivity to related proteins that contain the full (e.g. glicentin) or partial (e.g. mini-glucagon) glucagon sequence.

*"To address the need for a highly-specific glucagon assay, Merck developed two glucagon ELISAs."*

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## Preferred by researchers worldwide

Since their launch, Mercodia Glucagon ELISAs have been the assays of choice for researchers worldwide (Figure 3). They have been used in investigations studying pancreatic islets, diabetes therapies, obesity, NAFLD/NASH, nutrition, metabolomics, glucagon biosynthesis and secretion, among others (for a full reference list, see **Bibliography** on our web).

*“Mercodia Glucagon ELISAs have been the assays of choice for researchers worldwide.”*

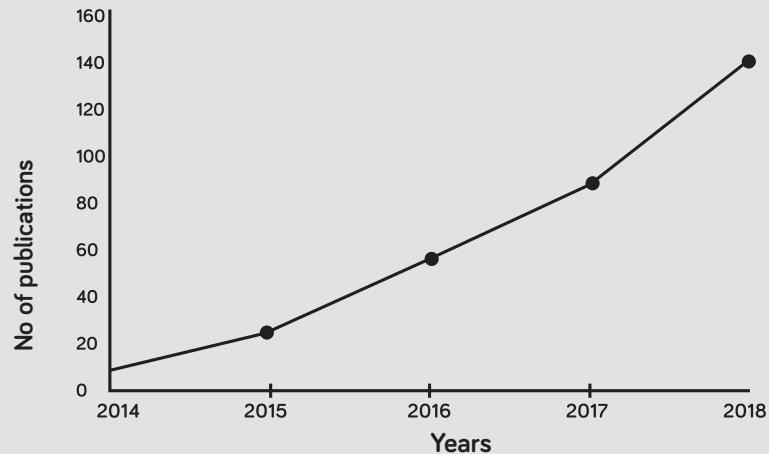


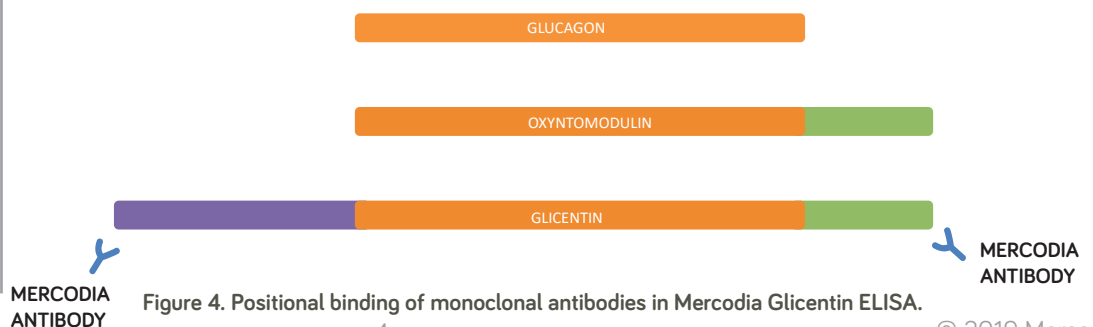
Figure 3. Accumulative use of Mercodia Glucagon ELISAs.

In a 2018 publication in the *Journal of Clinical Endocrinology and Metabolism*, German researchers analyzed glucagon dynamics during oral glucose tolerance tests (oGTTs) to investigate the role of hyperglucagonemia in type 2 diabetes<sup>9</sup>. Plasma glucagon was measured at five time-points of oGTT with Mercodia Glucagon ELISA (10-1271-01), in addition to a commercially-available radioimmunoassay (RIA). The researchers found that ELISA and RIA measurements gave different results.

In particular, suppression of plasma glucagon during oGTT was insufficiently represented in the RIA measurement. The authors argue that the sensitivity and specificity of ELISA for pancreatic glucagon is proven to be superior to RIA, and they thus exclusively used glucagon data measured by Mercodia ELISA throughout their investigations. In the discussion, the researchers stress the importance of highly-specific glucagon assays to study postprandial glucagon dynamics. They attribute the problem of RIA strongly underestimating glucagon suppression to cross-reactivity with other peptides cleaved from proglucagon, e.g. oxyntomodulin, glicentin and mini-glucagon: intestinal secretion of these peptides increases in the postprandial state, thereby masking glucagon suppression. They therefore advocate the use of sandwich ELISAs (Mercodia) with antibodies against both the N- and C-terminal end of the glucagon molecule since this dual approach circumvents the problem.

## Introduction of a specific glicentin ELISA

Until recently, glicentin (one of the proglucagon derivatives) has been difficult to measure due to the lack of commercial assays. But in 2014, Mercodia Glicentin ELISA (10-1273-01) was launched, thereby enabling research on the physiology of glicentin secretion<sup>8</sup>. This assay uses one antibody against the glucagon sequence, while the other recognizes the glicentin-related polypeptide sequence (Figure 4), allowing the quantitative determination of human glicentin in serum, EDTA plasma, and cell culture medium. Furthermore, it shows no cross-reactivity to glucagon, oxyntomodulin, mini-glucagon, GLP-1 or GLP-2.



## Previously unseen levels of glicentin

Studies have confirmed a stimulating effect of glucose ingestion on glicentin secretion compared to fasting concentrations and obese patients were shown to have significantly lower fasting serum concentrations of glicentin compared with lean subjects<sup>5,6</sup>. In the latter study (by Dr. Gribble and colleagues in Cambridge, UK), glicentin levels ranged between 10 and 100 pM.

This same study showed that glicentin levels in total gastrectomy patients (mimicking bariatric surgery) increased after oGTT to levels not seen previously in any patient group (around 400 pM)<sup>7</sup>. Oxyntomodulin levels were also found to increase (46 pM), but not to the same extent as glicentin.

When such high concentrations of glicentin (400 pM) appear in circulation, as in the case of bariatric surgery patients<sup>7</sup>, even a low cross-reactivity will significantly interfere in the measurement of glucagon, giving rise to falsely-elevated levels.

*“Before the launch of Mercodia Glicentin ELISA, it was not possible to accurately measure glicentin.”*

## Reached out to Mercodia

Mercodia Glucagon ELISA was launched in 2013 as a simultaneous protocol, meaning that the sample and the detection antibody are added together on the coated plate and then followed by a wash step, substrate addition and absorbance measurement. Before the launch of Mercodia Glicentin ELISA, it was not possible to accurately measure glicentin. Since the Cambridge researchers realized that high levels of glicentin after bariatric surgery could result in assay interference, they contacted Mercodia. The resulting discussions led to the development and validation of an alternative sequential protocol for Glucagon ELISA.

The antibodies used in this alternative assay are specifically directed against the N- and C-terminals of glucagon to prevent cross-reactivity to proteins containing the same amino acid residue sequence. Any cross-reactivity remaining is due to antibodies binding with less affinity, thus making it possible to decrease cross-reactivity even further by adding an additional wash step in the assay protocol. The introduction of this extra wash in our alternative protocol drastically lowered measured glucagon concentration in the post-oGTT samples of the gastrectomy group in the study. This occurred without substantially lowering measured glucagon levels in the fasting or post-oGTT samples in the control group, indicating that the glucagon measured post-oGTT in the gastrectomy group actually also contained a substantial amount of glicentin. This cross-reactivity was completely abolished with the alternative protocol. The authors conclude that caution should be exercised when interpreting glucagon results performed using standard assay protocols in patients after gastrectomy or Roux-en-Y reconstruction in whom glicentin and oxyntomodulin concentrations are particularly elevated.

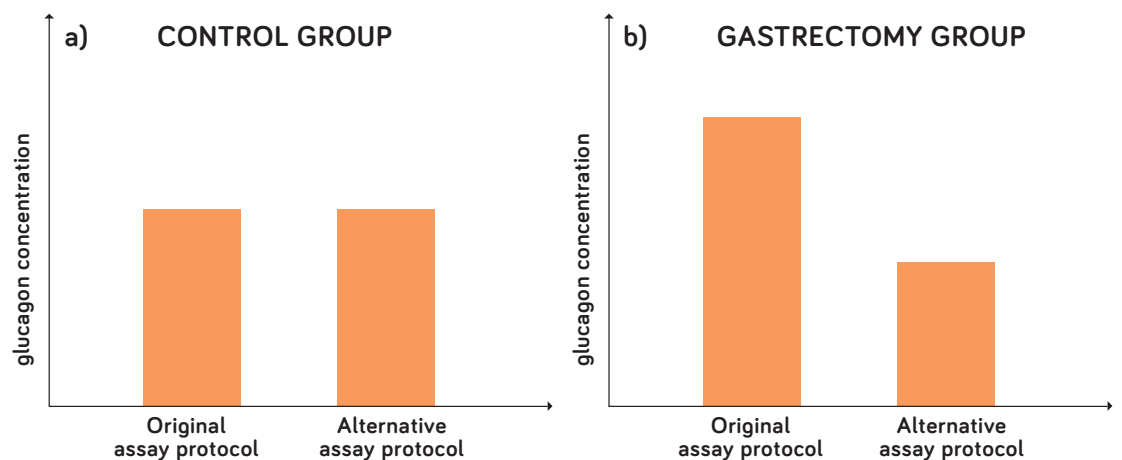


Figure 5. Schemati diagram of glucagon concentrations measured with different assay protocols for a) the control group and b) the gastrectomy group.

## Fruitful collaboration

The collaboration with Dr. Gribble's group in Cambridge resulted in the development of an alternative sequential protocol for Mercodia Glucagon ELISA (10-1271-01). This alternative offers an increased specificity that may be necessary under certain conditions, such as post-bariatric surgery, kidney disease and exogenous administration of pro-glucagon derivatives where the amounts of glicentin, proglucagon 1-61 and oxyntomodulin can be elevated to levels that might interfere with the assay<sup>6</sup>.

The alternative protocol is also validated with a fit-for-purpose approach. Its performance with respect to detection limit, cross-reactivity (Table 2), interference, dilutional linearity, parallelism, and sample correlation has been characterized.

*“The alternative protocol is also validated with a fit-for-purpose approach.”*

Table 2. Cross-reactivity of the alternative protocol for Glucagon ELISA.

	Cross-reaction (%)	Highest concentration tested (pmol/L)
<b>Glicentin</b>	0	600
<b>Oxyntomodulin</b>	0 2.1	40 600
<b>Glucagon 3-29</b>	0	1000
<b>Proglucagon 1-61</b>	0 2.1	25 1000

*...minimizes cross-reactivity from all proglucagon derivatives tested.”*

The alternative assay protocol minimizes cross-reactivity from all proglucagon derivatives tested, without significantly changing the sensitivity, interference, dilutional linearity, parallelism or sample correlation of the original protocol. Capability of detection is set to  $\leq 1$  pmol/L as determined with the methodology described in ISO11843-Part 4. The average recovery for dilutional linearity is 103% (99-108%), while parallelism shows an average recovery of 106% (98-120%). Finally, sample correlation between the alternative and the original assay protocol is excellent ( $R_2 > 0.98$ ).

To conclude, the high-specificity Mercodia Glucagon ELISA has, in collaboration with researchers from Cambridge, been proven effective when high levels of other proglucagon derivatives may be present. This interference would not have been detected without the introduction of the highly-specific Mercodia Glicentin ELISA.

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