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Evaluation of Mercodia Glucagon ELISA Kit

OBJECTIVE

Glucagon plays an important role in glucose homeostasis and therefore, in the management of diabetes. A majority of currently available assays to measure glucagon levels in plasma samples have been found to have a low level of specificity, due to varying levels of cross reactivity with other molecules and particularly with glicentin and oxyntomodulin which share significant amino acid sequence homology with glucagon. Additionally, if the antibodies used in the assays are not of high affinity, not only this further contributes to the lack of specificity but also significantly impacts the sensitivity and precision of the assays. Consequently, the data obtained by many of the available assays are not comparable thus impacting the interpretation and validity of the conclusions of published data. Recently, Mercodia has produced an ELISA kit based on the use of a pair of monoclonal antibodies directed against two different epitopes on the glucagon molecule. Mercodia claims the assay specifically measures only active glucagon (1-29). To further increase the specificity of the assay in samples when glicentin and oxyntomodulin are expected to be present in high concentration, an Extended Wash Protocol has been implemented to further increase the assay specificity by removal of cross reactivity with glicentin and oxyntomodulin.

We are evaluating the use of the Mercodia ELISA kit, and specifically, the Extended Wash Protocol for implementation of this assay in our laboratory. For this reason, we plan to validate the assay in our own laboratory to establish assay performance guidelines independent of the manufacturer claims for the product.

PLAN

The validation of Mercodia Glucagon kit will involve evaluation and verification of performance claims made by the manufacturer. The evaluation will involve comparison of results based on the Standard Protocol and Extended Wash Protocol.

- The standard curve will be constructed following the instructions provided in the kit insert.
- Assay sensitivity will be evaluated by analyzing zero calibrator multiple times within an assay.
- Reference interval will be determined in plasma samples of apparently healthy adults collected with addition of protease inhibitor cocktail.
- The intra-assay precision will be evaluated using three quality control samples provided by the manufacturer which are based on recombinant protein materials. The average glucagon level, standard deviation (SD) of mean, and coefficient of variation (CV) of analysis will be calculated.
- The same material will be used for the evaluation of the inter-assay precision. The average glucagon level, SD of mean, and CV of analysis will be calculated.

- The assay linearity will be evaluated by analyzing two serially diluted samples. The expected and observed results of the diluted samples will be plotted, and correlation between the results will be calculated.
- The analyte recovery will be evaluated using a set of plasma samples with different glucagon levels spiked with known amounts of glucagon . The spiked serum samples will be analyzed, and the expected and observed glucagon levels will be compared. This analysis will allow determination of analyte recovery in the analytical range.

The Mercodia glucagon assay kit will be deemed acceptable for implementation in the laboratory if:

- the assay curve, and signals for various analyte concentrations, are consistently within the indicated parameters,
- the assay sensitivity is verified as claimed by the manufacturer,
- the glucagon concentration in a set of apparently healthy individuals is within the normal range as indicated by the manufacturer,
- the glucagon assay meets the linearity requirement within the reportable range,
- the glucagon assay results meet the within-run precision requirement (CV less than 10%), and
- the results obtained on the quality control samples are within the range provided by the manufacturer.

MATERIALS USED

- Human glucagon ELISA Kit (Mercodia Catalog #10-1271-01, Lot #28689, Exp. 2021-01-31).
- QC Low, Medium and High glucagon controls (Mercodia Catalog #10-1286-01, Lot #27999, Exp. 2020-08-31)
- Plasma samples from 30 apparently healthy adults (no medical history taken)

ANALYSIS PROCEDURE

All the planned analyses will be performed using the protocol described in the kit insert (Attachment 1)
and repeated using the Extended Wash Protocol described in the document 34-0158 (Attachment 2)

RESULTS

STANDARD CURVE

Results of a typical standard curve for the Standard Protocol and Extended Wash Protocol, as obtained in our laboratory, are presented in Table 1 and Figures 1 and 2.

Table 1. Standard Curve

Glucagon (pM/L)	Mean OD Recorded		Calculated Glucagon Level (pM/L)	
	Standard Protocol	Extended Wash Protocol	Standard Protocol	Extended Wash Protocol
1.370	0.085	0.065	1.424	1.345
2.850	0.119	0.094	2.765	2.889
8.560	0.264	0.199	8.596	8.542
28.400	0.727	0.548	28.394	28.403
126.000	2.616	2.143	126.010	126.000

Figure 1: Standard Curve for Standard Protocol.

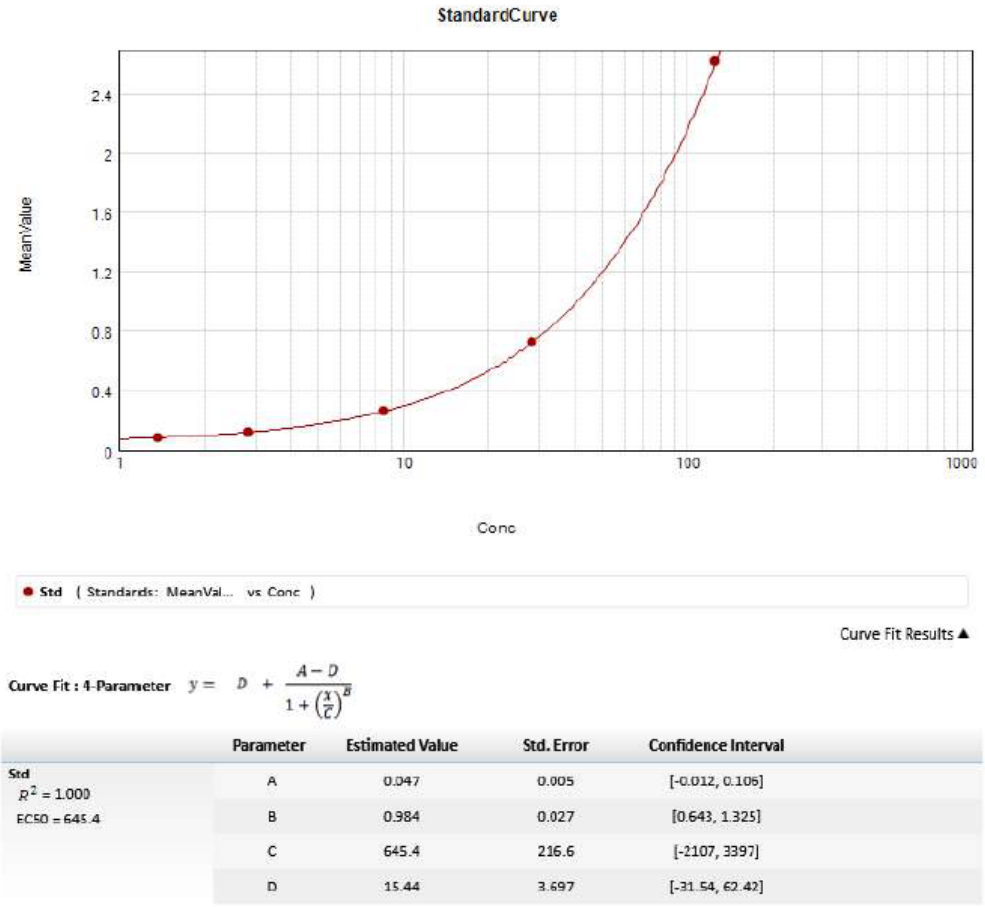
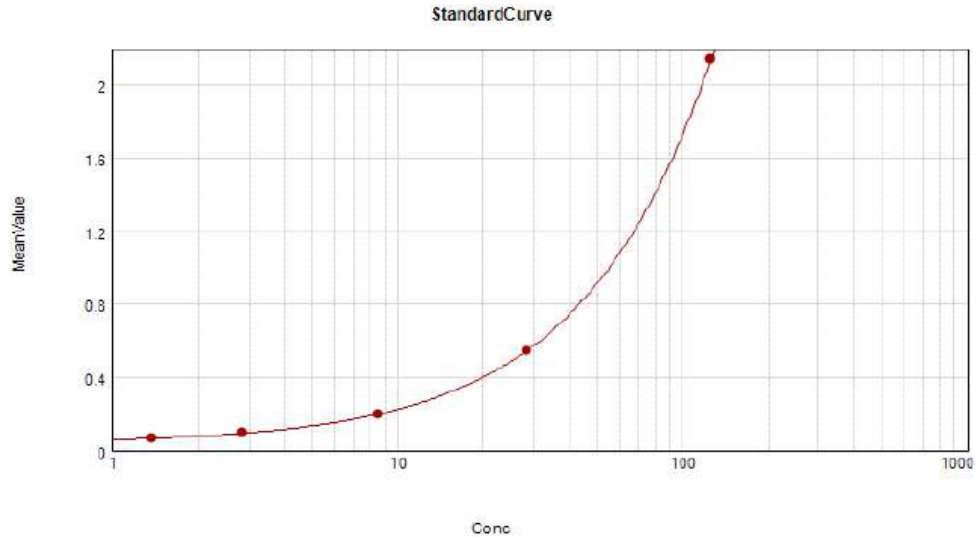


Figure 2: Standard Curve for Extended Wash Protocol.



Curve Fit Results ▲

Curve Fit : 4-Parameter $y = D + \frac{A-D}{1+\left(\frac{x}{C}\right)^B}$

	Parameter	Estimated Value	Std. Error	Confidence Interval
Std R^2 = 1.000 EC50 = 7482	A	0.037	0.002	[0.016, 0.059]
	B	0.961	0.013	[0.800, 1.123]
	C	7482	9565	[-1.14e+5, 1.29e+5]
	D	108.9	125.7	[-1489, 1706]

Conclusions: The standard curves in different plates were reproducible and produced appropriate signals for glucagon concentrations.

SENSITIVITY

Results of zero calibrator (assay blank) are presented in Table 2.

Table 2. Zero Calibrator Replicates

	Standard Protocol	Extended Wash Protocol
	Optical Density (OD)	Optical Density (OD)
Mean	**0.049375**	**0.0435**
SD	**0.001204159**	**0.004082483**
Mean OD + 3 SD	**0.0530**	**0.0557**
	0.0510	0.0560
	0.0510	0.0400
	0.0520	0.0440
	0.0500	0.0390
	0.0500	0.0480
	0.0500	0.0430
	0.0490	0.0420
	0.0480	0.0450
	0.0490	0.0430
	0.0490	0.0440
	0.0480	0.0430
	0.0490	0.0440
	0.0480	0.0420
	0.0490	0.0420
	0.0480	0.0380
	0.0490	0.0430
Assay sensitivity	**0.85 pM/L**	**1.10 pM/L**
Mercodia sensitivity	**1.00 pM/L**	**1.00 pM/L**

Conclusions: The assay sensitivity stated by Mercodia is confirmed.

VERIFICATION OF REFERENCE RANGE

Analyses were performed on samples from 30 apparently healthy volunteers and are presented in Table 3. Eleven of the 30 samples were frozen for one month, whereas 19 samples were frozen for approximately 5 years, and thus mimicking the RISE Study sample conditions.

Table 3. Reference Range of Glucagon in Human Plasma Samples

	Standard Protocol				Extended Wash Protocol			
	Result 1	Result 2	Mean	CV (%)	Result 1	Result 2	Mean	CV (%)
1	5.77	5.57	5.67	2.5	5.52	5.31	5.42	2.8
2	8.56	7.99	8.28	4.9	7.44	7.88	7.66	4.1
3	14.97	14.88	14.92	0.5	13.02	13.14	13.08	0.7
4	10.08	9.76	9.92	2.3	9.11	9.46	9.29	2.7
5	6.55	6.27	6.41	3.1	6.70	6.64	6.67	0.6
6	22.90	22.40	22.65	1.6	18.59	19.00	18.79	1.5
7	6.94	6.93	6.93	0.0	6.82	6.50	6.66	3.4
8	3.52	3.74	3.63	4.4	3.52	3.52	3.52	0.1
9	13.90	13.72	13.81	0.9	13.60	13.33	13.46	1.4
10	2.56	2.56	2.56	0.1	2.51	2.52	2.51	0.4
11	18.91	19.65	19.28	2.7	17.87	18.05	17.96	0.7
12	5.64	5.65	5.65	0.1	5.41	5.51	5.46	1.4
13	21.73	21.74	21.74	0.0	19.29	19.09	19.19	0.8
14	10.05	10.10	10.08	0.3	9.29	9.17	9.23	0.9
15	10.68	10.24	10.46	3.0	8.93	8.78	8.85	1.2
16	8.14	7.99	8.04	1.8	5.65	5.82	5.74	2.1
17	5.66	5.45	5.57	3.1	4.91	4.96	4.93	0.7
18	6.49	6.33	6.41	1.7	6.30	6.34	6.32	0.5
19	10.37	9.86	10.12	3.6	8.53	9.05	8.79	4.1
20	7.20	7.23	7.21	0.3	6.36	6.56	6.46	2.3
21	4.51	4.38	4.44	2.1	3.95	3.90	3.93	1.0
22	23.63	24.86	24.25	3.6	21.52	21.62	21.57	0.3
23	21.19	20.61	20.90	2.0	12.92	12.65	12.79	1.5
24	5.33	5.25	5.29	1.0	4.97	5.66	5.32	9.1
25	3.22	3.09	3.16	2.9	3.09	3.15	3.12	1.4
26	5.24	5.14	5.19	1.3	4.98	5.20	5.09	3.0
27	5.97	6.04	6.01	0.9	4.88	4.88	4.88	0.1
28	8.65	8.52	8.58	1.1	8.69	8.69	8.69	0.0
29	7.43	7.63	7.53	1.8	6.94	6.85	6.9	0.9
30	10.47	10.04	10.26	2.9	8.25	8.27	8.26	0.2
	Average CV %			**1.88**	**Average CV %**			**1.66**
	Range			**2.56 - 24.25 pM/L**	**Range**			**2.51 - 21.57 pM/L**
	Median			**7.78 pM/L**	**Median**			**6.78 pM/L**

Mercodia: Central 95% reference range <1.5 - 18 pM/L, median value of 6.5 pM/L.

Conclusions: On the basis of the limited data from the current analysis, the reference range can be considered verified.

ANALYTE RECOVERY UPON DILUTION (Assay Linearity)

Two samples were used for evaluation of the linearity of results. The first sample (L1) was prepared by spiking the High Glucagon Control with Standard 5. The spiked sample was analyzed undiluted and at 1/4, 1/16, 1/32 and 1/64 dilutions. All the dilutions were prepared in zero calibrator.

A second sample (L2) was prepared by using the highest standard (S5), which was diluted at 1/2, 1/8, 1/32, 1/64, and 1/128 in zero calibrator. Various dilutions were analyzed in the Standard Protocol and the Extended Wash Protocol. This selection of samples was necessitated because there were no human samples available with very high glucagon levels.

Results of various dilutions for L1 and L2 samples are presented in Table 5.

Table 5: Linearity

Standard Protocol			
Dilution	Observed Glucagon Level (pM/L)	Expected Glucagon Level (pM/L)	Percent Recover
Sample L1			
Undil	92.01	92.01	100.0
1:4	25.03	23.00	108.8
1:16	6.40	5.75	**111.3**
1:32	2.77	2.88	**96.2**
1:64	0.70	1.44	48.7
1:128	ND*		
Sample L2			
Undil	121.53	121.53	100.0
1:4	66.49	60.77	**109.4**
1:16	16.49	15.19	108.5
1:32	3.56	3.80	**93.7**
1:64	1.16	1.90	60.8
1:128	ND*		

Extended Wash Protocol			
Dilution	Observed Glucagon Level (pM/L)	Expected Glucagon Level (pM/L)	Percent Recover
Sample L1			
Undil	93.13	93.13	100.0
1:4	23.68	23.28	101.7
1:16	5.60	5.82	96.3
1:32	2.78	2.91	**95.5**
1:64	1.87	1.46	**128.7**
1:128	0.45	0.73	61.7
Sample L2			
Undil	123.37	123.37	100.0
1:4	67.77	61.69	**109.9**
1:16	16.22	15.42	105.2
1:32	3.41	3.86	**88.6**
1:64	1.82	1.93	94.3
1:128	0.64	0.96	66.2

*ND = No glucagon detected

Mercodia: 81 - 96%

Conclusions: The linearity obtained in our laboratory for the Standard Protocol is 96.2 - 111.3% for sample L1 and 93.7 - 109.4% for sample L2. An unacceptable recovery was found at 1:64 dilution for both samples. For the Extended Wash Protocol, our recovery is 95.5 - 128.7% for sample L1 and 88.6 - 109.9% for sample L2, up to 1:64 dilution. Therefore, we confirm the optimal linearity of the Extended Wash Protocol but not for the Standard Protocol.

ANALYTE RECOVERY UPON ADDITION (Assay Parallelism)

For the evaluation of the analyte recovery, three human plasma samples (U1-U3) were spiked to different levels using purified glucagon material received as assay standards (S3, S4 or S5). The glucagon levels of pre-spiked samples (U1-3 and S3-S5) are presented in Table 6. A total of six spiked samples (P1-P6) were prepared. The plan of the spiking of three plasma samples and results of the spiked samples (P1-P6) is presented in Table 7.

Table 6. Glucagon Levels (pM/L) of Three Plasma Samples (U1-U3) and the Standards (S3-S5)

Sample	Standard Protocol	Extended Wash Protocol
U1	5.49	4.91
U2	5.99	4.46
U3	10.15	8.96
S3	9.75	8.49
S4	31.52	30.83
S5	131.42	131.99

Table 7. Analyte Recovery in Glucagon-Spiked Samples

Pools	Composition	Standard Protocol			Extended Wash Protocol		
		Observed	Expected	Recovery	Observed	Expected	Recovery
P1	100 uL U1 + 200 uL S5	79.29	89.44	88.65	93.95	89.63	104.82
P2	65 uL U2 + 150 uL S4	19.19	23.01	83.39	21.93	22.37	98.02
P3	150uL U3 + 200 uL S3	9.46	9.92	95.32	9.28	8.69	**106.80**
P4	100 uL U2 + 125uL S3	6.73	7.85	85.69	7.06	6.90	102.35
P5	100 uL U2 + 100 uL S5	58.50	68.70	**85.15**	63.39	68.73	**92.23**
P6	75 uL U3 + 120 uL S5	93.01	84.77	**109.72**	89.84	84.67	106.11

Mercodia: Range of recovery 96 - 101% for the Standard Protocol and 98 - 120% for the Extended Wash Protocol.

Conclusions: The analyte recovery upon addition obtained in our laboratory for both protocols validates the results obtained by Mercodia.

INTRA-ASSAY VARIATION

The single set of quality control samples provided by the manufacturer did not have enough volume to evaluate the intra-assay precision. For this reason, the determination was performed using three in-house collected plasma samples with low, medium, and high glucagon levels and the data are presented in Table 4.

TABLE 4. Intra-assay Precision

	Standard Protocol			Extended Wash Protocol		
	Low (pM/L)	Medium (pM/L)	High (pM/L)	Low (pM/L)	Medium (pM/L)	High (pM/L)
	2.52	4.33	33.28	1.80	3.06	29.60
	2.48	4.47	34.07	1.88	3.21	29.98
	2.40	4.29	34.58	1.86	3.20	31.16
	2.37	4.30	33.93	1.90	3.21	30.38
	2.44	4.25	33.53	1.84	3.19	31.51
	2.46	4.27	34.08	1.97	3.24	30.06
	2.44	4.32	34.30	1.58	3.22	30.64
	2.41	4.32	41.61*	1.80	3.05	30.79
	2.95	4.27	34.27	1.85	3.10	31.41
	2.37	4.41	34.11	1.85	3.17	31.04
	2.35	4.36	34.22	1.82	3.07	31.15
	2.49	4.36	34.19	1.77	3.13	30.84
	2.49	4.45	34.16	1.80	3.04	31.07
	2.45	4.42	34.18	2.22	3.03	31.58
	2.56	4.38	35.14	1.82	2.73	31.39
	2.70	4.33	34.96	1.83	3.12	31.23
AVG	**2.49**	**4.35**	**34.20**	**1.85**	**3.11**	**30.86**
ST DEV	**0.15**	**0.07**	**0.46**	**0.13**	**0.12**	**0.59**
% CV	**5.92**	**1.53**	**1.36**	**6.89**	**3.98**	**1.91**

Note: *Result for an outlier high glucagon sample result was excluded from calculation.

Mercodia: Reported CV % values for the low, medium, and high concentration quality control samples are 5.1%, 3.6%, and 3.3%, respectively, for the Standard Protocol.

Conclusions: The within-run precision obtained in our lab by the Standard Protocol using individual plasma samples at low, medium, and high concentration of glucagon, are similar to or lower than the CVs obtained by Mercodia using recombinant quality control samples. The within-run precision is therefore validated. A higher imprecision was obtained for the Extended Wash Protocol, but no data is available from Mercodia.

EVALUATION OF THE QUALITY CONTROL SAMPLES

We received six ELISA kits but only a single set of quality control samples. The originally received set of QC samples were analyzed in plates 1 and 2 on November 13, 2018 and on plates 3 and 4 on November 15, 2018. The new set of requested QC samples were analyzed in plates 5 and 6 on November 28, 2018.

Plate	Standard Protocol			Extended Wash Protocol		
	Low QC (Lot 27641) pM/L	Medium QC (Lot 27642) pM/L	High QC (Lot 27643) pM/L	Low QC (Lot 27641) pM/L	Medium QC (Lot 27642) pM/L	High QC (Lot 27643) pM/L
1	4.417	14.466	45.194			
2				4.843	14.474	42.017
3	4.193	12.471	39.981			
4				4.104	12.686	38.987
5	4.716	15.641	43.546			
6				4.401	14.49	43.648
Average	**4.442**	**14.193**	**42.907**	**4.449**	**13.883**	**41.550**
SD	**0.262**	**1.602**	**2.665**	**0.371**	**1.036**	**2.365**

Mercodia: Reported average values for the low, medium, and high level quality control materials are 4.9 pM/L (range 4.17 - 5.64 pM/L), 14.7 pM/L (range 12.9 - 16.5 pM/L), and 43.6 pM/L (range 38.4 - 48.8 pM/L), respectively, for the Standard Protocol.

Conclusions: The values obtained on the three quality control materials in both protocols are very close to the average values obtained by Mercodia and therefore, the quality of the validation analyses is confirmed.

METHOD COMPARISON

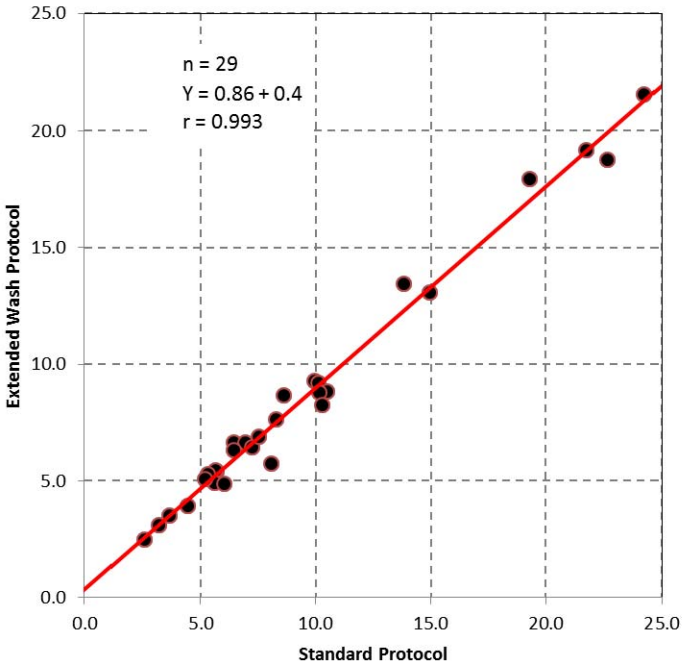
Results of 30 samples analyzed by the Standard Protocol and by the Extended Wash Protocol are presented in Table 8.

Table 8. Comparison of Glucagon Level (pM/L)

Sample	Standard Protocol	Extended Wash Protocol	Bias (pM/L)	Bias (%)	Absolute Bias (%)
1	5.67	5.42	0.25	4.42	4.42
2	8.28	7.66	0.62	7.44	7.44
3	14.92	13.08	1.84	12.34	12.34
4	9.92	9.29	0.63	6.38	6.38
5	6.41	6.67	-0.26	-4.06	4.06
6	22.65	18.79	3.86	17.03	17.03
7	6.93	6.66	0.27	3.94	3.94
8	3.63	3.52	0.11	3.05	3.05
9	13.81	13.46	0.35	2.51	2.51
10	2.56	2.51	0.05	1.87	1.87
11	19.28	17.96	1.32	6.85	6.85
12	5.65	5.46	0.19	3.33	3.33
13	21.74	19.19	2.55	11.72	11.72
14	10.08	9.23	0.84	8.38	8.38
15	10.46	8.85	1.61	15.35	15.35
16	8.04	5.74	2.30	28.59	28.59
17	5.57	4.93	0.64	11.52	11.52
18	6.41	6.32	0.09	1.47	1.47
19	10.12	8.79	1.33	13.15	13.15
20	7.21	6.46	0.75	10.45	10.45
21	4.44	3.93	0.52	11.61	11.61
22	24.25	21.57	2.68	11.04	11.04
23	20.90	12.79	8.11	**38.82***	**38.82***
24	5.29	5.32	-0.03	-0.50	0.50
25	3.16	3.12	0.04	1.11	1.11
26	5.19	5.09	0.10	1.87	1.87
27	6.01	4.88	1.13	18.77	18.77
28	8.58	8.69	-0.11	-1.22	1.22
29	7.53	6.90	0.63	8.41	8.41
30	10.26	8.26	2.00	19.46	19.46
		Average	**1.15**	**8.15**	**8.55**

***Result of outlier excluded**

Figure 3: Sample Correlation



Conclusions: The glucagon results obtained by the two assay protocols are highly correlated. With the exception of three samples, all the values obtained by the Extended Wash Protocol are lower than those obtained by the Standard Protocol, with an absolute bias ranging from 1.11% to 28.59% (excluding as an outlier the samples with a bias of 38.82%).

OVERALL CONCLUSIONS

The results of the validation of the Mercodia ELISA for measuring glucagon in human samples show that both assay protocols, in general, meet our established acceptability criteria. The comparison of the results obtained on samples from healthy individuals show a decrease in glucagon levels obtained by the Extended Wash Protocol as compared to the Standard Protocol. This observation needs further evaluation with a larger number of samples because it is not clear if the observed decrease is due to removal of glucagon by the extended wash or reflect removal of cross-reacting molecules as claimed by Mercodia.

Attachments:

1. Product insert for Glucagon ELISA 10-1271-01 v1_0
2. TN34-0158 Alternative Sequential Protocol in Mercodia Glucagon ELISA v4

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Date

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