Glucagon Kit

Procedure for the Radioimmunoassay of
Human Plasma Glucagon

For In Vitro Diagnostic Use

MP Biomedicals™

Cat No. 07-152101 (50 TUBES)

TABLE OF CONTENTS

I. INTRODUCTION

The role of glucagon, a polypeptide hormone secreted from the alpha cells of the pancreas, has become of great interest to investigators in the field of glucose metabolism. Glucagon (pancreatic) is a polypeptide hormone consisting of 29 amino acids with a molecular weight of 3500. Due to its size and composition, glucagon has a very short biological half-life of about 5 minutes and is extremely susceptible to proteolytic enzyme degradation.[1,2]

Glucagon plays an antagonistic role with insulin in the regulation of blood glucose levels.[3] Thus, its presence acts as a hormone of energy release, raising blood sugar levels by glycogenolysis and gluconeogenesis, opposing insulin's action of energy storage primarily by glycogenesis. Much evidence suggests that diabetic patients not only demonstrate a deficiency of insulin release, but also manifest inappropriately elevated glucagon levels. Further consideration of the importance of glucagon in the pathogenesis of Diabetes Mellitus and of diabetic ketoacidosis has demonstrated that Somatostatin, a hypothalamic factor which inhibits glucagon release, can delay or prevent the development of ketoacidosis in juvenile onset diabetics deprived of insulin.[4,5] In addition to its role in the diabetic syndrome, elevated plasma glucagon levels have been reported in patients with hyperglycemic diabetes, chronic renal failure and glucagonoma.[6,7,8,9]

A variety of factors can influence the glucagon levels of normal, healthy individuals. Low glucose levels in the blood caused by fasting will elevate glucagon, while high glucose levels will decrease glucagon. Stress, exercise and certain amino acids will also alter normal values.[9,10,11,12,] For these reasons, dynamic testing such as glucose tolerance or fasting provide the clinician more accurate information than one single test result.[13]

Finally, due to the instability of glucagon in blood, special collection techniques and assay conditions are required for this test.

II. EXPECTED NORMAL VALUES

The following normal values were obtained using EDTA plasma samples.

Normal* 25-250 pg/mL

Laboratories not using EDTA plasma must establish their own normals.

* Standardized against WHO 69/194 glucagon.

III. PRINCIPLE OF RIA

Radioimmunoassay (RIA) is the term applied to the measurement of the concentration of antigen molecules using a radioactive label that quantitates the amount of antigen (i.e., hormone) by determination of the extent to which it combines with its antibody.

In the assay, a limited amount of specific antibody (Ab) is reacted with the corresponding hormone (*H) labeled with a radioisotope. Upon addition of an increasing amount of the hormone (H), a correspondingly decreasing fraction of *H added is bound to the antibody. After separation of the bound from the free *H by various means, the amount of radioactivity in one or both of these two fractions is evaluated and used to construct a standard curve against which the unknown samples are measured.

IV. SPECIMEN COLLECTION AND HANDLING

Glucagon is relatively unstable and must be handled with some care if accurate results are to be obtained. The patient should be relaxed and care must be taken to avoid anxiety, as it may elevate the patient's Glucagon level.

EDTA PLASMA ONLY ! ! !

Prior to drawing blood, chill purple capped (EDTA) vacutainer to 0°C - 4°C. Draw blood into the vacutainer and invert gently to mix contents. If drawn blood cannot be centrifuged immediately, place tubes in an ice bath. Because of the short half-life of glucagon in whole blood, samples should be centrifuged within sixty (60) minutes from drawing time. Refrigerated centrifugation is recommended, although a short centrifugation at 20°C should not appreciably affect the sample. Plasma stored at 4°C should be used within four (4) hours. Plasma stored at -20°C can be stored for one (1) week. For longer storage, add Trasylol™ and store at -72°C. Samples may only be thawed once.

The use of Heparin and Sodium Citrate as anticoagulants must be avoided.

V. EQUIPMENT AND REAGENTS REQUIRED

A. Pipettors and/or pipets that can accurately and precisely deliver the required volumes. (100µL, 200µL, 1 mL).

B. Gamma counter.

C. Laboratory vortex mixer.

D. Test tube rack with ice bath large enough to accommodate the rack.

E. Centrifuge (refrigerated preferred) capable of 950-1050 x g.

F. Absorbent paper for blotting or an aspiration device.

G. Refrigerated water bath or a refrigerator capable of maintaining 0 to 4°C.

H. Glass test tubes for assay.

VI. REAGENTS (FOR IN-VITRO DIAGNOSTIC USE ONLY)

Reagent	Color Code	No. of Vials	Volume (50 Tube Kit)
Ultra-pure water	Tan	1	50 mL
Anti-glucagon	Yellow	1	11 mL*
Standards (25-2000 pg/mL)	Green	7	1.0 mL*
Standard (0 pg/mL)	Green	1	3.0 mL*
Controls	Grey	2	1.0 mL*
Second Antibody	Red	1	5.5 mL
PEG Reagent	White	1	10 mL
Glucagon-^{125}I	Blue	1	5.5 mL*

* Supplied in Lyophilized Form.

A. Anti-glucagon ANTI: Porcine glucagon-HSA was used as the antigen to generate antiserum in rabbits. The antiserum is titered to bind 28-40% of the Glucagon ^{125}I in the absence of non-radioactive glucagon. Prior to use, reconstitute with 11 mL of ultra-pure water and allow to stand 15 minutes at 0 to 4 °C. After use, store below -15°C. Thaw only once.

Storage: Lyophilized at 2 to 8°C
Reconstituted below -15°C

Stability: Refer to the expiration date on the vial.

B. Glucagon Standards STD 1-7: Seven porcine glucagon ($^{1-29}$) standards are provided at the following target concentrations: 25, 50, 100, 250, 500, 1000 and 2000 pg/mL. Please refer to the standard vial label for exact standard concentrations. Prior to use, reconstitute with 1.0 mL of ultra-pure water, and allow to stand 15 minutes at 0 to 4°C. After use, store below -15°C. Thaw only once.

Storage: Lyophilized at 2 to 8°C
Reconstituted below -15°C

Stability: Refer to the expiration date on the vial.

C. Glucagon Zero Standard STD 0: A standard containing no glucagon. Prior to use, reconstitute with 3.0 mL of ultra pure water and allow to stand 15 minutes at 0 to 4°C. After use, store below -15°C. Thaw only once.

Storage: Lyophilized at 2 to 8°C
Reconstituted below -15°C

Stability: Refer to the expiration date on the vial.

D. Glucagon Controls CONTROL 1-2: A high and a low concentration of porcine glucagon has been added to the zero standard for controls. Prior to use, reconstitute with 1.0 mL of ultra-pure water and allow to stand 15 minutes at 0 to 4°C. After use store below -15°C. Thaw only once.

Storage: Lyophilized at 2 to 8°C
Reconstituted below -15°C

Stability: Refer to the expiration date on the vial.

E. Precipitating Antiserum [ANTI 2nd]: Goat anti-rabbit gamma globulins in 0.01M phosphosaline buffer pH 7.5. 0.1 mL, together with an equal amount of 30% PEG 6000 (see F below), will precipitate all the antibody bound antigen.

Storage: Store below -15°C

Stability: Refer to expiration on kit vial.

(To minimize bacterial growth, freeze in convenient aliquots. Freezing and thawing will not affect the viability of the precipitating antisera)

F. 30% polyethylene glycol [PEG] (PEG 6,000) in 0.01M phosphosaline buffer, pH 7.5.

Storage: Room temperature.

Stability: Refer to expiration on kit vial.

G. Ultra-pure water [DILUENT H2O]: Ultra purified water is provided to reconstitute all lyophilized reagents in this kit.

H. Glucagon [^{125}I TRACER] (porcine glucagon $^{1-29}$): This radioactive material contains less than 1µCi per vial on the date of shipment. 0.1 mL of this radioactive material will provide approximately 20,000 cpm at 75% counter efficiency on the calibration day. This material is provided lyophilized and should be reconstituted with 5.5 mL of ultra pure water and allowed to stand 15 minutes at 0 to 4°C before use. After use, store below -15°C. Thaw only once.

Storage: Lyophilized at 2 to 8°C

Reconstituted below -15°C

PLEASE OBSERVE THE FOLLOWING PRECAUTIONS WHEN HANDLING THIS RADIOACTIVE MATERIAL

I. This radioactive material may be received, acquired, possessed, and used those licensed to do so. It is intended for in-vitro clinical or laboratory tests not involving internal or external administration of the materials. Thus, the possession, use and transfer of the radiation herein is subject to the regulations of, and with a general license from, the U.S. NRC or the State with which the NRC has entered into agreement for the exercise of regulatory authority.

J. Immediately upon receipt of this kit, check for breakage and verify contents as per the packing list. Should there be breakage or questions regarding this kit's contents, please immediately notify your supplier by telephone.

K. Kit reagents should be stored and used only at clean, designated work stations of the laboratory. Although exposure to radiation from the small amount of radioactive material supplied is negligible, it is good practice to designate a storage area at least 10 feet from any work station. Furthermore, persons under the age of 18 should not be permitted to handle radioactive material or enter an area where it is present.

L. Should there be spillage of any radioactive material, the following clean-up procedure is recommended. While wearing rubber gloves, blot the spillage with a paper towel. This contaminated towel should be disposed of as radioactive waste. Wash the affected area with a detergent, then rinse gloves with water, tear to prevent further use and discard as ordinary waste.

The pipetting of radioactive material by mouth should not be permitted. Smoking, eating, or drinking while performing tests involving radioactive material should be prohibited. Lastly, persons handling radioactive material should wash their hands immediately after handling and prior to leaving the laboratory.

VII. LIMITATIONS, PRECAUTIONS AND GENERAL COMMENTS

A. Strict adherence to the Protocol is required for reliable and optimum performance.

B. Samples must be collected in EDTA. The use of other anticoagulants may give erroneous results.

C. Due to the instability of glucagon, samples must constantly be kept at 2 to 4°C and are stable for approximately 3 hours at this temperature. Otherwise, they should be stored below -20°C and assayed within 7 days. If the samples are to be assayed after 7 days, a proteolytic enzyme inhibitor, such as Aprotinin (Trasylol™) should be added to the sample prior to freezing and the sample should be stored below -72°C.

D. Lipemic or grossly hemolyzed samples should not be used, since they may give inaccurate results.

E. The kit reagents and materials are intended as an integral unit. Do not mix various lots of any component reagent within an individual run.

F. A standard curve must be established for each run.

G. The reagents provided in this kit are for IN-VITRO DIAGNOSTIC USE ONLY and are intended for the quantitation of human plasma samples. Researchers doing animal work are advised to establish their own normal ranges.

H. The use of plastic assay tubes should be avoided.

VIII. ASSAY PROCEDURE

A. Prior to assay, reconstitute the lyophilized reagents (except the Glucagon^{125}I) with the ultra pure water provided, mix gently and let sit 15 minutes at 0 to 4°C, or allow frozen reagents to thaw in a 0 to 4°C environment.

B. Set up the assay in duplicate in GLASS test tubes. Either of 2 assay set up techniques may be employed:

- Place a test tube rack in an ice water bath. All reagents and samples should similarly be placed in the ice water bath,

OR

- If you can pipette the assay in 10 minutes or less, place your test tube rack on your bench while keeping the reagents and samples in an ice bath. Assay must be pipetted within 10 minutes for accurate results.

1. Add ultra pure water, Glucagon standards, controls, samples and antiserum as indicated in the protocol. Vortex the tubes thoroughly by touching several times on a vortex mixer and incubate for 6 hours at 4±2°C.

2. After the 6 hour incubation (above), add 0.1 mL of Glucagon^{125}I to all tubes (15 minutes prior to use, reconstitute with 5.5 mL, mix gently and let sit for 15 minutes at 0 to 4°C or let thaw at 0 to 4°C). Vortex thoroughly by touching several times on a vortex mixer and incubate at least 16 hours at 4±2°C.

3. After the 16 hour incubation, add 0.2 mL of second antibody - PEG mixture to all tubes. (COMBINE EQUAL QUANTITIES OF SECOND ANTIBODY AND PEG AND VORTEX THOROUGHLY. COMBINE ONLY THE AMOUNT NEEDED FOR EACH RUN).

4. Vortex all tubes (thoroughly) immediately after the addition of second antibody- PEG mixture.

5. Add 1 mL of cold distilled water (not provided in kit) to each tube.

6. Centrifuge at 2300 - 2500 rpm (1000 x g) for 15 minutes. Aspirate or decant the supernatant. If decanting, blot the rim of test tubes on absorbent paper towel before turning upright.

7. Count the precipitate in a gamma counter. A counting time of at least 2 minutes is recommended.

IX. PROTOCOL

Quantitative Glucagon Incubation 6 + 16 hrs. at 4 ± 2°C

Tube No.	Water (mL)	Std. or Sample (mL)	Anti-Glucagon (mL)	Gluc. ^{125}I	Description	% Bound	Results
1,2	0.2	0.2		0.1	Blank 0 pg/mL		
3,4	0	0.2	0.2		0 pg/mL		
5,6					25 pg/mL		
7,8					50 pg/mL		
9,10					100 pg/mL		
11,12					250 pg/mL		
13,14					500 pg/mL		
15,16					1000 pg/mL		
17,18					2000 pg/mL		
19,20					Low Control		
21,22					High Control		
23,24					Sample		
25,26					Sample		
27,28					Sample		
29,30	▼	▼	▼	▼	Sample		

X. CALCULATIONS

A. Average the counts of all duplicate tubes. Subtract the averaged blank (NSB) counts form the average above. This yields the corrected counts. Divide the corrected counts by the corrected zero standard counts to obtain the percent bound (B/Bo).

Formula:

$$B/Bo = \frac{\overline{CPM}\text{ (sample)} - \overline{CPM}\text{ (Blank NSB)}}{\overline{CPM}\text{ (0 standard)} - \overline{CPM}\text{ (Blank NSB)}}$$

$\overline{CPM}$ = Average counts of duplicate tubes.

Sample = Standard, control, or unknown sample.

Blank (NSB) = Blank tube (also known as non-specific binding tube).

0 Standard = 0 tube (also known as 100% binding tube).

B. Plot percent bound versus the concentration of Glucagon for all the standards, (25 - 2000 pg/mL). This yields the standard curve. Sample values may then be read directly from this curve.

C. Although the curve is linear on log-logit paper, it is erroneous to extrapolate a value for a patient sample that binds either higher or lower than the standard curve.

Sample Calculation:

$$\%\ B/Bo = \frac{(25\text{ pg/mL Standard}) - \text{Blank})}{(\text{0 standard}) - (\text{Blank})} \times 1000$$

$$= \frac{(6226) - (966)}{(6442) - (966)} \times (100)$$

$$= \frac{5260}{5476} \times 100$$

25 pg/mL = 96%

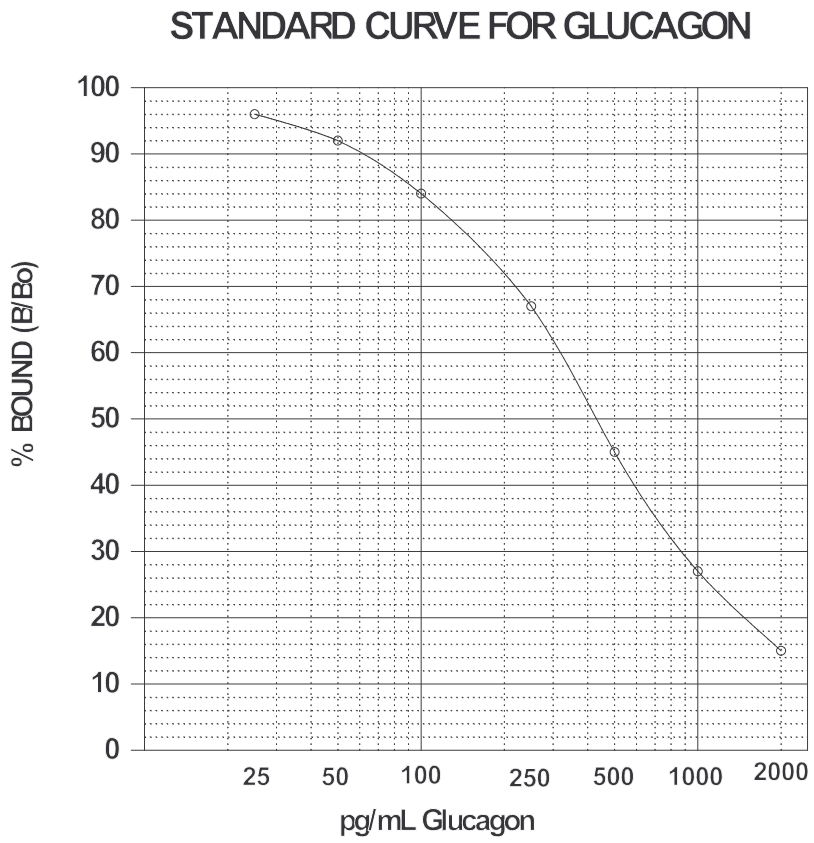
D. Sample Assay:

These calculations are for example only. The user must construct a standard curve each time the assay is run.

Tube Description	CPM	Average CPM	Ave-Blank CPM	% Bound	Result
Blank (NSB)	973 959	966			
0	6481 6403	6442	5476		
25	6171 6282	6226	5260	96	
50	5997 5996	5997	5031	92	
100	5649 5528	5588	4622	84	
250	4680 4644	4662	3696	67	
500	3390 3506	3448	2482	45	
1000	2316 2581	2448	1482	27	
2000	1787 1806	1796	830	15	
Control I	5847 5793	5820	4854	89	
Control II	4971 5041	5006	4040	74	

XI. **STANDARD CURVE FOR GLUCAGON**

This curve serves only as an example. Patient sample values must not be derived from it.



XII. SPECIFICITY OF THE ANTISERUM

The following materials have been checked for cross reactivity. The percentages indicate cross-reactivity at 50% displacement compared to pancreatic glucagon.

COMPOUND	% CROSS REACTION
Pancreatic Glucagon	100
Gut Glucagon	0.0013
Porcine Insulin	0.0005
Porcine Gastrin	0.0005
Human ACTH	<0.0005
Glucagon $^{22-29}$	<0.0005
Glucagon $^{19-29}$	<0.0005
Glucagon $^{30-37}$	<0.0005
Preproglucagon $^{78-107}$	<0.0005

XIII. PERFORMANCE CHARACTERISTICS

A. Parallelism (linearity of dilution)

Sample No.	Baseline	1:2	1:3	1:4	1:5
1	-----	540 x 2=	340 x 3=	280 x 4=	160 x 5=
	900	1080	1020	1120	800
2	440	250 x 2=	175 x 3=	125 x 4=	85 x 5=
		500	525	500	425
3	200	120 x 2=	78 x 3=	58 x 4=	45 x 5=
		240	234	232	225
4	100	60 x 2=	41 x 3=	33 x 4=	25 x 5=
		120	123	132	125

B. Recovery :

	Glucagon added (pg/mL)	Glucagon expected (pg/mL)	Glucagon obtained (pg/mL)	% Recovery
Sample A	-----	-----	33	-----
	25	58	70	121
	50	83	78	94
	125	158	150	95
	250	283	250	88
	500	533	540	101
	1000	1033	1100	106
				101
Sample B	-----	-----	45	-----
	25	70	64	91
	50	95	78	82
	125	170	150	88
	250	295	270	92
	500	545	530	97
	1000	1045	1025	98
				91
Sample C	-----	-----	70	-----
	25	95	105	111
	50	120	140	117
	125	195	200	103
	250	320	330	103
	500	570	530	93
	1000	1070	1300	122
				108
Sample D	-----	-----	90	-----
	25	115	110	96
	50	140	140	100
	125	215	210	98
	250	340	390	115
	500	590	660	112
	1000	1090	1200	110
				105

C. Inter-Assay Variation (n=12)

	Control I	Control II
	130	427
	144	448
	99	416
	91	404
	120	408
	123	432
	118	410
	108	416
	104	420
	110	414
	114	407
	108	393
mean	114.1	416.3
S.D.	14.2	14.4
C.V.	12.4%	3.5%

D. Intra-Assay Variation (n=12)

	Control A	Control B
	49	104
	47	97
	45	109
	45	93
	63	117
	50	117
	47	100
	52	98
	60	103
	59	102
	59	103
	57	127
mean	52.8	105.8
S.D.	6.5	9.9
C.V.	12.3%	9.4%

XIV. REFERENCES

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Hergestellt von:
Fabriqué par:
Prodotto da:
Fabricado por:

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07814521.1
QER NO.: Q03-177
12/04