

Instruction

# EURIA-Glucagon

Glucagon radioimmunoassay

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***For Research Use Only. Not for use in diagnostic procedures.***

**REF** RB 310 RUO



100

## INTRODUCTION

Glucagon is a 29 amino acids straight chain peptide produced in the pancreatic  $\alpha$ -cells (1,2). Glucagon is cleaved out from preproglucagon with 159 amino acids. The amino acid sequence of glucagon is found in glicentin, a 69 amino acid peptide (3). Glicentin has been proposed to be a biosynthetic intermediate for pancreatic and gut glucagon.

Glucagon is involved in carbohydrate, fat and protein metabolism. Basal amounts of glucagon are essential for the maintenance of normoglycemia and a physiological role for glucagon is to prevent hypoglycemia. Increases in the plasma glucagon level affect glucose production first by stimulating a transient phase of glycogenolysis and then a prolonged period of glyconeogenesis (4,5).

A sustained increase in the glucagon level continues to modulate hepatic glucose production (6).

Glucagon also plays a role in the amino acid metabolism. Elevation of glucagon in plasma decreases amino acids whereas glucagon deficiency increases amino acids (7,8,9). The amino acid sequence of human pancreatic glucagon: His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr.

## PHYSIOLOGICAL CONSIDERATIONS

Glucagon is involved in carbohydrate, fat and protein metabolism. Basal amounts of glucagon are essential for the maintenance of normoglycemia and a physiological role for glucagon is to prevent hypoglycemia.

Pancreatectomy do not cause totally glucagon deficiency. However, the concentrations in plasma are significantly lower than in normals (7,10).

Since glucagon in diabetics has been found elevated absolutely or relatively to insulin, it has been proposed that glucagon contributes essentially to the development of the hyperglycemia and keto acidosis found in diabetes (11,12,13). Elevated levels of glucagon in plasma are found in patients with A-cell tumors (8).

**Normal level of glucagon in plasma after 12 hours fasting:** <60 pmol/L (obtained with this method). It is recommended that users establish reference ranges for the populations served by their own laboratories.

## PRINCIPLE OF THE METHOD

The intended use of these reagents is for assay of glucagon in human plasma. Glucagon in plasma is assayed by the competitive radioimmunoassay using a rabbit antiserum raised against a glucagon-albumin conjugate. Glucagon in standards and samples compete with  $^{125}\text{I}$ -labelled glucagon in binding to the antibodies in a two steps incubation.

$^{125}\text{I}$ -glucagon binds in a reverse proportion to the concentration of glucagon in standards and samples. Antibody-bound  $^{125}\text{I}$ -glucagon is separated from the unbound fraction using double antibody solid phase. The radioactivity of the bound fraction is measured in a gamma counter.

The antiserum used in this assay shows less than 0.1% cross reaction with gut-GLI (14). For professional use within a laboratory. The result shall not be used for clinical diagnosis or patient management.

## PRECAUTIONS

### ***For research use only. Not for use in diagnostic procedures.***

As the regulations may vary from one country to another, it is essential that the person responsible for the laboratory is familiar with current local regulations, concerning all aspects of radioactive materials of the type and quantity used in this test.

This kit contains components of human origin. They have been tested by immunoassay for hepatitis B surface antigen, antibodies to HCV and for antibodies to HIV-1 and HIV-2 and found to be negative. Nevertheless, all recommended precautions for the handling of blood derivatives, should be observed.

This kit contains  $^{125}\text{I}$  (half-life: 60 days), emitting ionizing X (28 keV) and  $\gamma$  (35.5 keV) radiations. Steps should be taken to ensure the proper handling of the radioactive material, according to local and/or national regulations. Only authorized personnel should have access to the reagents.

The following precautions should be observed when handling radioactive materials:

- Radioactive material should be stored in specially designed areas, not normally accessible to unauthorized personnel.
- Handling of radioactive material should be conducted in authorized areas only.
- Care should be exercised to prevent ingestion and contact with the skin and clothing. Do not pipette radioactive solutions by mouth.
- Drinking, eating or smoking should be prohibited where radioactive material is being used.
- Hands should be protected by gloves and washed after using radioactive materials.
- Work should be carried out on a surface covered by disposable absorbing material.
- Spills of radioactive material should be removed immediately, and all contaminated materials disposed as radioactive waste. Contaminated surfaces should be cleaned with a detergent.

The reagents in this kit contain sodium azide. Contact with copper or lead drain pipes may result in the cumulative formation of highly explosive azide deposits. On disposal of the reagents in the sewerage, always flush with copious amounts of water, which prevents metallic azide formation. Plumbing suspected of being contaminated with these explosive deposits should be rinsed thoroughly with 10% sodium hydroxide solution.

## COMPOSITION OF THE REAGENT KIT

The reagents provided in each kit is sufficient for 100 tubes.

### 1. Anti-glucagon (Reagent A)

Rabbit antiserum raised against porcine glucagon, conjugated to human serum albumin. Lyophilized in 5.0 mL 2.0 M glycine buffer, pH 8.8, 2.5% human serum albumin, 0.5% sodium azide and aprotinin (Trasylo<sup>®</sup> or equivalent). Reconstitution in 52 mL distilled water. The reconstituted reagent contains 500 KIU aprotinin /mL. Colour: Yellow.

### 2. <sup>125</sup>I-Glucagon (Reagent B)

Total radioactivity: 0.75  $\mu$ Ci or 28 KBq at reference date. Produced by iodination of synthetic human glucagon. HPLC-purified, monoiodinated.

Specific activity: 1700-2100  $\mu$ Ci/nmol (62-77 MBq/nmol). Lyophilized in 5.0 mL 2.0 M glycine buffer, pH 8.8, 2.5% human serum albumin, 0.5% sodium azide and aprotinin (Trasylo<sup>®</sup> or equivalent). Reconstitution in 52 mL distilled water. The reconstituted reagent contains 500 KIU aprotinin /mL. Colour: Blue.

### 3. Double antibody solid phase (Reagent C)

Anti-rabbit-Ig coupled to cellulose particles in 0.01 M phosphate buffer pH 6.8 with 0.25% human serum albumin, 0.045% NaCl, 0.05% NaN<sub>3</sub>, 0.185% EDTA and 0.05% Tween 80. Volume: 11 mL suspension.

### 4. Assay diluent (Reagent D)

50 mL of 0.2 M glycine buffer, pH 8.8, containing 0.25% human serum albumin, 0.05% sodium azide and 500 KIU aprotinin /mL.

To be used for the preparation of glucagon working standards and instead of antiserum in non-specific binding control tubes.

### 5. Glucagon standard (Reagent E)

5.00 mL standard. Concentration: 300 pmol/L (1044 pg/mL) of synthetic human glucagon. Lyophilized in 0.2 M glycine buffer, pH 8.8, containing 0.25% human serum albumin, 0.05% sodium azide and 50<sup>®</sup>/mL. Reconstitution in 5.00 mL distilled water.

### 6. Controls (Reagent F-G)

Lyophilized controls. 2.00 mL of each control after reconstitution. The glucagon concentrations of the controls are given on the labels of the vials.

Contains 0.05% sodium azide.

## REAGENTS AND EQUIPMENT REQUIRED BUT NOT PROVIDED

Distilled water  
Disposable test tubes of polystyrene: 11-13x55 mm  
Pipettes with disposable tips: 200 and 500  $\mu$ L  
Glass pipettes 1.00 mL and 5.00 mL (for standard preparation)  
Vortex mixer  
Centrifuge, refrigerated, giving a minimum of 1700 x g.  
Gamma counter

## REAGENT PREPARATION AND STORAGE

Store all reagents at 2-8° C before reconstitution and use.  
The water used for reconstitution of the lyophilized reagents should be distilled in an all-glass apparatus or be of corresponding purity. Dissolve the contents in the vials by gentle inversion and avoid foaming.  
The stability for each reagent is found on the label of the vial. For the lyophilized reagents the expiry date is valid for the unreconstituted reagents. The reconstituted reagents are stable for 10 weeks (or to the expiry date for the labelled glucagon) when stored according to the instructions below.

### **Reagent A: Anti-Glucagon**

Reconstitute with 52 mL of distilled water.  
Store at 2-8° C.

### **Reagent B: <sup>125</sup>I-Glucagon**

Reconstitute with 52 mL of distilled water.  
Store at -18° C or lower if reused.

### **Reagent C: Double antibody solid phase**

Ready for use. Stir continuously during pipetting this reagent.  
Store at 2-8° C.

### **Reagent D: Assay diluent**

Ready for use. Store at 2-8° C.

### **Reagent E: Glucagon standard**

Reconstitute with 5.00 mL distilled water.  
Store at -18° C or lower if reused.

### **Reagent F-G: Controls**

Reconstitute with 2.00 mL distilled water. Store at -18° C or lower if reused.

## SPECIMEN COLLECTION

Vein blood is collected in 10mL test tubes containing EDTA and aprotinin 5000 KIU/mL (Trasylol® or equivalent). The sample is cooled in an ice-bath immediately. Plasma is separated by centrifugation (refrigerated centrifuge is preferred). The plasma should be frozen within 2 hours and stored at -18° C or lower until assayed. Repeated freezing and thawing must be avoided.

## ASSAY PROCEDURE

Reconstitute the reagents as specified. Accuracy in all pipetting steps is essential. All tests (standards, samples and controls) should be performed in duplicate.

For an overview of the assay procedure see page 10.

A complete assay includes:

**Standard (St-tubes):** 7 concentrations: 0, 4.7, 9.4, 18.8, 37.5, 75, 150 pmol/L  
(= 0, 16.3, 32.6, 65.3, 131, 261, 522 pg/mL).

**Controls (C-tubes):** Two different controls with known concentrations of glucagon for quality control.

### **Samples (S-tubes)**

Tubes for determination of the **non-specific binding (NSB-tubes)**

Tubes for determination of the **total radioactivity added (TOT-tubes)**

1. Reconstitute the reagents according to the instructions.
2. Prepare the glucagon working standards by dilution of the 300 pmol/L standard (Reagent E) with the assay diluent (Reagent D) according to the following:
  - a/ 1.00 mL standard 300 pmol/L + 1.00 mL assay diluent = 150 pmol/L
  - b/ 1.00 mL standard 150 pmol/L + 1.00 mL assay diluent = 75 pmol/L
  - c/ 1.00 mL standard 75 pmol/L + 1.00 mL assay diluent = 37.5 pmol/L
  - d/ 1.00 mL standard 37.5 pmol/L + 1.00 mL assay diluent = 18.8 pmol/L
  - e/ 1.00 mL standard 18.8 pmol/L + 1.00 mL assay diluent = 9.4 pmol/L
  - f/ 1.00 mL standard 9.4 pmol/L + 1.00 mL assay diluent = 4.7 pmol/L
  - g/ Assay diluent = 0 pmol/LStore the standard solutions a-g and Reagent E at -18° C or lower if reused.
3. Pipette 200 µL of the standards a-g, samples and controls in their respective tubes (duplicates).
4. Pipette 200 µL of the assay diluent in the NSB-tubes for standard.

5. Pipette 500  $\mu\text{L}$  anti-glucagon (Reagent A) in all tubes except the NSB- and TOT-tubes.
6. Pipette 500  $\mu\text{L}$  assay diluent (Reagent D) in the NSB-tubes.
7. Vortex-mix and incubate for 20-24 hours at 2-8° C.
8. Pipette 500  $\mu\text{L}$   $^{125}\text{I}$ -Glucagon (Reagent B) in all tubes. The TOT-tubes are sealed and kept aside.
9. Vortex-mix and incubate for 20-24 hours at 2-8° C.
10. Add 100  $\mu\text{L}$  double antibody solid phase (Reagent C) to all tubes except the TOT-tubes. Stir continuously during pipetting this reagent
11. Vortex-mix and incubate for 30-60 minutes at 2-8° C.
12. Centrifuge the tubes for 15 minutes at +4° C (1700 x g).  
**Note:** The correct centrifugation force is important for accurate performance.
13. Decant the liquid immediately after centrifugation.  
**Note:** The accurateness and coherency in handling of supernatants are crucial for the assay precision.
14. Count the radioactivity of the pellets in a gammacounter. The counting time should be at least 2 minutes.

### **CALCULATION OF RESULTS**

1. Subtract the average count rate (CPM) of the non-specific binding tubes for standard from the count rate (CPM) of the replicates of the standard tubes, the sample tubes and control tubes.
2. A standard curve is generated by plotting the bound CPM (in CPM or % B/TOT) against the concentration of the glucagon standards.
3. Interpolate the glucagon concentrations in the samples and controls from the generated standard curve.
4. The standard curve and the calculation of the concentrations of the samples can be done by a suitable computer program. A spline algorithm may be used.
5. A typical standard curve is shown on page 11.  
A typical run is shown on page 12.

## QUALITY CONTROL

In order to enable the laboratory to completely monitor the consistent performance of the assay, the following important factors should be checked.

### 1. The found concentrations of the controls

The found concentrations of the controls (Reagent F and G) should be within the limits given on the labels of the vials.

### 2. Total counts

Counts obtained should approximate the expected CPM when adjusted for counter efficiency and radioactive decay. The content of <sup>125</sup>I-glucagon in this kit will give 10.500 CPM (-5, +30%) at the reference date (counting efficiency: 80%).

### 3. Maximum binding (Bo/TOT)

Calculate for each assay the % bound radioactivity in the zero-standard:

$$\frac{B_0}{TOT} \times 100 \%$$

### 4. Non-specific binding (NSB/TOT)

Calculate for each assay the % non-specific binding:  $\frac{NSB}{TOT} \times 100$

The non-specific binding should be less than 6%.

### 5. Slope of standard curve

For example, monitor the 80, 50 and 20% points of the standard curve for run to run reproducibility.

**ASSAY CHARACTERISTICS**

**Sensitivity**

The lowest detectable concentration in the assay is 3 pmol/L. This figure corresponds to a decrease in binding of 2 x SD of the bound radioactivity in the zero-standard.

**Precision**

Intra assay variation:

<u>Level</u>	<u>Coefficient of variation (%CV)</u>	<u>N</u>
16.4 pmol/L	8.1	30
60.1 pmol/L	4.5	30

Total variation (sum of intra- and inter assay variation):

<u>Level</u>	<u>Coefficient of variation (%CV)</u>	<u>N</u>
25.4 pmol/L	6.8	6
22.0 pmol/L	7.4	6
23.0 pmol/L	8.3	5
73.9 pmol/L	3.9	6
97.9 pmol/L	5.6	6

**Accuracy**

The recovery was 97.6% when known amounts of glucagon were added to plasma samples.

**Specificity**

The following cross reactions have been found:

<u>Peptide</u>	<u>Cross reaction</u>
Glucagon, pancreatic, human	100.0%
Gut GLI	<0.1%
Secretin	<0.02%
Cholecystokinin -39	<0.02%
Vasoactive intestinal peptide	<0.02%
Gastric inhibitory peptide	<0.02%
GLP1	<0.1%
Oxyntomodulin	<0.1%

**Correlation**

Euria-Glucagon assay correlates with WHO 69/194 standard.

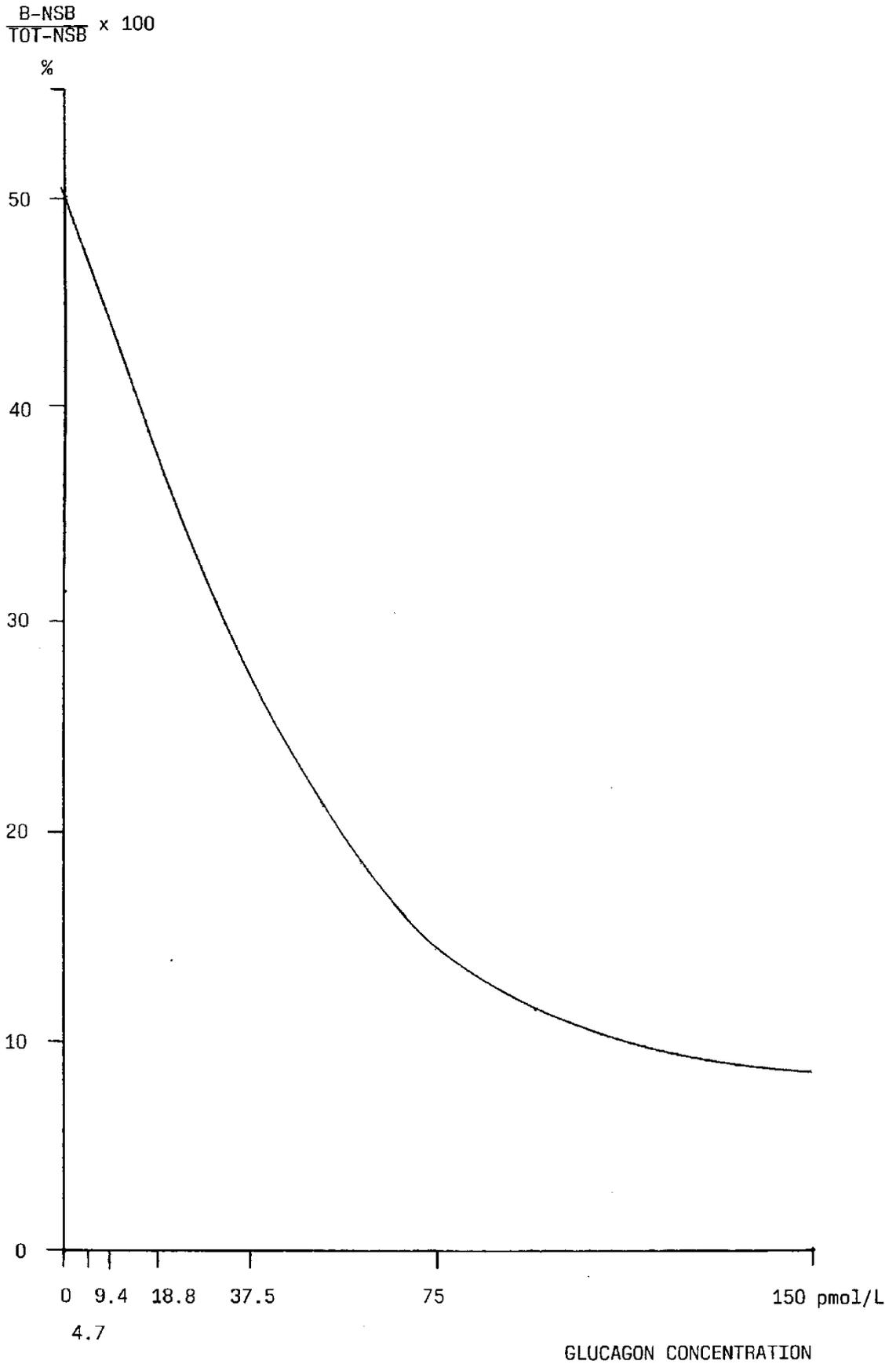
**Interference**

Samples displaying cloudiness, hemolysis, hyperlipemia or containing fibrin may give inaccurate results.

**OUTLINE OF THE RIA PROCEDURE**

Type of tubes	Tube no	Standard sample or control	Anti-glucagon (A)	Assay diluent (D)		<sup>125</sup> I-glucagon (B)		Double antibody solid phase (C)	
TOT NSB <sub>st</sub>	1-2	-	-	-	Vortex-mix and incubate for 20-24 hours at 2-8° C.	500 µL	Vortex-mix and incubate for 20-24 hours at 2-8° C.	-	Vortex-mix and incubate for 30-60 min. at 2-8° C. Centrifuge 15 min. at 1700 x g at +4° C. Decant and count the radioactivity of the precipitates.
Stand 0	3-4	200 µL	-	500		500 µL		100 µL	
Stand 4.7	5-6	200 µL	500 µL	-		500 µL		100 µL	
Stand 9.4	7-8	200 µL	500 µL	-		500 µL		100 µL	
Stand 18.8	9-10	200 µL	500 µL	-		500 µL		100 µL	
Stand 37.5	11-12	200 µL	500 µL	-		500 µL		100 µL	
Stand 75	13-14	200 µL	500 µL	-		500 µL		100 µL	
Stand 150	15-16	200 µL	500 µL	-		500 µL		100 µL	
Control (F)	17-18	200 µL	500 µL	-		500 µL		100 µL	
Control (G)	19-20	200 µL	500 µL	-		500 µL		100 µL	
Sample 1 etc.	21-22	200 µL	500 µL	-		500 µL		100 µL	
	23-24	200 µL	500 µL	-	500 µL	100 µL			

EXAMPLE OF A GLUCAGON STANDARD CURVE



**TYPICAL DATA FOR GLUCAGON STANDARD CURVE AT ACTIVITY REFERENCE DATE**

Tube no	Type of tube	Concentration pmol/L	CPM (raw)	$\frac{B}{TOT} \times 100$
1	NSB <sub>st</sub>	-	684	5.5%
2	"	-	653	5.3%
3	TOT	-	12301	$\frac{B-NSB}{TOT-NSB} \times 100$
4	"	-	12347	
5	St	0.0	6253	50.7%
6	"	"	6203	
7	St	4.7	5827	50.3%
8	"	"	5775	
9	St	9.4	5267	47.3%
10	"	"	5384	
11	St	18.8	4661	46.9%
12	"	"	4729	
13	St	37.5	3379	37.8%
14	"	"	3310	
15	St	75	1777	38.4%
16	"	"	1760	
17	St	150	1050	27.4%
18	"	"	1081	
				26.9%
				14.4%
				14.3%
				8.5%
				8.8%

**Control parameters**

$\frac{B_0}{TOT} \times 100$  : 48.0 %

$\frac{NSB}{TOT} \times 100$  : 5.4 %

ED 80 : 14.5 pmol/L

ED 50 : 39.8 pmol/L

ED 20 : 100 pmol/L

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**SYMBOLS USED ON LABELS**

	<p>Batch code.</p>
	<p>Catalogue number.</p>
	<p>Use by date.</p>
	<p>Temperature limit.</p>
	<p>Date of manufacture.</p>
	<p>Contains radioactive substances.</p>
	<p>Biological risks.</p>
	<p>Consult instructions for use.</p>
	<p>Manufacturer.</p>
	<p>Contains sufficient for 100 tests.</p>

<b>REAG</b>   <b>A</b>   <b>Ab</b>	Anti-Glucagon.
<b>REAG</b>   <b>B</b>   <b>Ag</b>   <sup>125</sup> I	<sup>125</sup> I-Glucagon.
<b>REAG</b>   <b>C</b>   <b>DASP</b>	Double antibody solid phase.
<b>REAG</b>   <b>D</b>   <b>DIL</b>   <b>AS</b>	Assay diluent.
<b>REAG</b>   <b>E</b>   <b>CAL</b>   <b>300</b>	Glucagon standard 300 pmol/L.
<b>REAG</b>   <b>F</b>   <b>CONTROL</b>	Control, level 1 (low).
<b>REAG</b>   <b>G</b>   <b>CONTROL</b>	Control, level 2 (high).

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