

Glucagon ELISA

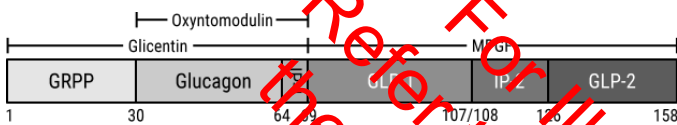


AL-157-i

INTENDED USE

The Glucagon Enzyme Linked Immunosorbent Assay (ELISA) kit provides materials for the quantitative measurement of Glucagon in human plasma and other biological fluids. This assay is intended for *in vitro* diagnostic use.

SUMMARY AND EXPLANATION



Glucagon and Oxyntomodulin, a peptide hormone secreted by the alpha cells of pancreas, share identical amino acid sequence in the N-terminal 29 aa. Glucagon is a 29-amino acid polypeptide processed from proglucagon in pancreatic alpha cells.¹ In intestinal L-cells proglucagon is cleaved into glicentin, corresponding to proglucagon residues no. 1-69. Glicentin can further be processed into oxyntomodulin, corresponding to proglucagon residues no. 33-69. These peptides are released simultaneously upon stimulation. Glucagon has shown to have an effect opposite to that of insulin, i.e. it raises blood glucose levels. It causes the liver to convert glycogen into glucose, which is then released into the blood stream.²⁻⁴ During hypoglycemia, glucagon secretion offers a protective feedback mechanism, defending the organism against damaging effects of glucose deficiency in the brain and nerves.⁵

PRINCIPLE OF THE TEST

The Glucagon ELISA is a quantitative two-step sandwich type immunoassay. In the first step Calibrators, Controls and unknown samples are added to Glucagon antibody coated microtiter wells and incubated with biotinylated Glucagon antibody. After the first incubation and washing, the wells are incubated with streptavidin horseradish peroxidase conjugate (SHRP). After the second incubation and washing step, the wells are incubated with substrate solution (TMB). After TMB incubation, an acidic stopping solution is added. In principle, the antibody-biotin conjugate binds to the solid phase antibody-antigen complex which in turn binds to the streptavidin-enzyme conjugate. The antibody-antigen-biotin conjugate-SHRP complex bound to the well is detected by enzyme-substrate reaction. The degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurement at 450 nm as primary test filter and 630 nm as reference filter. The absorbance measured is directly proportional to the concentration of Glucagon in the samples and calibrators.

MATERIALS SUPPLIED

CAL-157A Glucagon Calibrator A (Lyophilized)

One vial, labeled A, containing concentration of approximately 0 pg/mL Glucagon in protein-based buffer and ProClin™ 300. Store unopened at 2 to 8°C until the expiration date. Reconstitute calibrator A with 1 mL deionized water. Solubilize, mix well and use after reconstitution.

CAL-157B - CAL-157G Glucagon Calibrators B thru G (Lyophilized)

Six vials, labeled B-G, containing concentrations of approximately 7-300 pg/mL Glucagon in heat treated serum with non-mercury preservative. Refer to **calibration card** for exact concentrations. Store unopened at 2 to 8°C until the expiration date. Reconstitute calibrators B-G with 1 mL deionized water. Solubilize, mix well and use after reconstitution.

Note: The calibrator concentration in the kit is traceable to WHO 69/194.

CTR-157-I & CTR-157-II Glucagon Controls I & II (Lyophilized)

Two vials, labeled Levels I and II containing low and high Glucagon concentrations in heat treated serum with non-mercury preservative. Refer to **calibration card** for exact control ranges. Store unopened at 2 to 8°C until the expiration date. Reconstitute control Levels I and II with 1 mL deionized water. Solubilize, mix well and use after reconstitution.

SPD-157 Glucagon Sample Diluent

One vial, containing concentration of approximately 0 pg/mL Glucagon in heat treated serum with non-mercury preservative. Store unopened at 2 to 8°C until the expiration date. Reconstitute with 1 mL deionized water. Solubilize, mix well and use after reconstitution.

PLT-157 Glucagon Coated Microtitration Strips

One strip holder, containing 12 strips and 96 microtitration wells with Glucagon antibody immobilized to the inside wall of each well. Store at 2-8°C until expiration date in the resealable pouch with a desiccant to protect from moisture.

BCT-157 Glucagon Bio in Conjugate Ready-To-Use (RTU)

One bottle, 12 mL, containing biotinylated anti-Glucagon antibody in protein-based buffer with a non-mercury preservative. Store at 2-8°C until expiration date.

SAR-157 Glucagon Streptavidin-Enzyme Conjugate—Ready-to-Use

One amber bottle, 12 mL, containing streptavidin-HRP (horseradish peroxidase) in a protein-based buffer and a non-mercury preservative. Store undiluted at 2-8°C until expiration date.

TMB-100 TMB Chromogen Solution

One bottle, 12 mL, containing a solution of tetramethylbenzidine (TMB) in buffer with hydrogen peroxide. Store at 2 to 8°C until expiration date.

STP-100 Stopping Solution

One bottle, 12 mL, containing 0.2 M sulfuric acid. Store at 2 to 30°C until expiration date.

WSH-100 Wash Concentrate A

One bottle, 60 mL, containing phosphate buffer saline solution with a nonionic detergent. Store at 2 to 30°C until expiration date. Dilute 25-fold with deionized water prior to use.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Microplate reader capable of absorbance measurement at 450 nm, 405nm and 630 nm.
2. Microplate orbital shaker.
3. Microplate washer.
4. Semi-automated/manual precision pipette to deliver 10–250 μL .
5. Vortex mixer.
6. Deionized water.
7. Disposable 12 x 75 mm culture tubes.
8. Tight fitting 12 x 75 mm tube racks.

WARNINGS AND PRECAUTIONS**For *in vitro* diagnostic use.**

The following precautions should be observed:

- a) Follow good laboratory practices.
- b) Use personal protective equipment. Wear lab coats and disposable gloves when handling immunoassay materials.
- c) Handle and dispose of all reagents and material in compliance with applicable regulations.
- d) If external package is damaged, inspect the components inside for any other damage. Do not use if the components are damaged.

WARNING: Potential Biohazardous Material

This reagent may contain heat treated serum or materials used in conjunction with human source materials. Handle all reagents and patient samples at a Biosafety Level 2, as recommended for any potentially infectious human material in the Centers for Disease Control/National Institutes of Health manual "Biosafety in Microbiological and Biomedical Laboratories," 5th Edition, 2020.⁶

WARNING: Potential Chemical Hazard

Some reagents in this kit contain ProClin™ 300 and Sodium azide as a preservative. ProClin™ 300 and Sodium azide in concentrated amounts are irritants to skin and mucous membranes.

For further information regarding hazardous substances in the kit, please refer to the MSDS, either at AnshLabs.com or by request.

SAMPLE COLLECTION AND PREPARATION

- a) K_2 EDTA plasma is the recommended sample type.
- b) Li-Heparin plasma can also be used. However, glucagon in Li-Heparin plasma and serum samples may be more sensitive to storage conditions and freeze-thaw cycles. EDTA plasma tubes may result in 20-30% higher glucagon values. Use of one tube type is highly recommended for use when analyzing data.
- c) Sample handling, processing, and storage requirements depend on the brand of blood collection tube that you use. Please reference the manufacturer's instructions for guidance. Each laboratory should determine the acceptability of its own blood collection tubes and serum separation products. Addition of aprotinin to EDTA plasma or serum samples will not improve stability. EDTA Plasma tubes with and without DPP-IV inhibitors may result in 20-30% higher glucagon values.
- d) Avoid storing samples at room temperature or 2-8°C for longer than 2 hours.
- e) Samples should be stored at -80°C to avoid loss of bioactivity and contamination.
- f) Avoid assaying lipemic, hemolyzed or icteric samples.
- g) Avoid repeated freezing and thawing of samples. Thaw samples no more than 3 times.
- h) For shipping, place specimens in leak proof containers in biohazard specimen bags with appropriate specimen identification and test

requisition information in the outside pocket of the biohazard specimen bag. Follow DOT and IATA requirements when shipping specimens.⁸

PROCEDURAL NOTES

1. A thorough understanding of this package insert is necessary for successful use of the Glucagon ELISA assay. It is the user's responsibility to validate the assay for their purpose. Accurate results will only be obtained by using precise laboratory techniques and following the package insert.
2. A calibration curve must be included with each assay.
3. Bring all kit reagents to room temperature ($23 \pm 2^\circ\text{C}$) before use. Thoroughly mix the reagents before use by gentle inversion. Do not mix various lots of any kit component and do not use any component beyond the expiration date.
4. Use a clean disposable pipette tip for each reagent, calibrator, control or sample. Avoid microbial contamination of reagents, contamination of the substrate solutions with the HRP conjugates. The enzyme used as the label is inactivated by oxygen, and is highly sensitive to microbial contamination, sodium azide, hypochlorous acid and aromatic chlorohydrocarbons often found in laboratory water supplies. Use deionized water.
5. Incomplete washing will adversely affect the outcome and assay precision. To minimize potential assay drift due to variation in the substrate incubation time, care should be taken to add the substrate solution into the wells. Avoid exposure of the reagents to excessive heat or direct sunlight during storage and incubation.

PREPARATION OF REAGENTS

1. **Glucagon Calibrators A–G and Glucagon Controls I & II:** Tap and reconstitute Glucagon Calibrators A-G with 1.0 mL deionized water. Solubilize for 10 minutes, mix well and use after reconstitution.
2. **Glucagon Sample Diluent:** Tap and reconstitute Glucagon Sample Diluent with 1.0 mL deionized water. Solubilize for 10 minutes, mix well and use after reconstitution.
3. **Wash Solution:** Dilute wash concentrate 25-fold with deionized water. The wash solution is stable for one month at room temperature ($23 \pm 2^\circ\text{C}$) when stored in a tightly sealed bottle.
4. **Microtitration Wells:** Select the number of coated wells required for the assay. The remaining unused wells should be placed in the resealable pouch with a desiccant. The pouch must be resealed to protect from moisture.

ASSAY PROCEDURE

Allow all specimens and reagents to reach room temperature ($23 \pm 2^\circ\text{C}$) and mix thoroughly by gentle inversion before use. Calibrators, controls, and unknowns should be assayed in duplicate.

NOTE: All samples reading higher than the highest calibrator should be mixed and diluted in the 0 pg/mL reconstituted Glucagon Sample Diluent prior to assay.

1. Reconstitute Glucagon Calibrators A-G and Glucagon Controls I & II each with **1.0 mL** deionized water. Solubilize for **10 minutes**, Mix well.
2. Reconstitute Glucagon sample diluent with **1.0 mL** deionized water. Solubilize for **10 minutes**, Mix well.
3. Label the microtitration strips to be used.
4. Pipette **50 μL** of the Calibrator, Controls and Unknowns to the appropriate wells.
5. Add **100 μL** of the Antibody-Biotin Conjugate RTU to each well using a repeater pipette.
6. Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **2 hours** at room temperature ($23 \pm 2^\circ\text{C}$).

- Aspirate and wash each strip **5 times** with Washing Solution (**350 μ L/per well**) using an automatic microplate washer.
- Add **100 μ L** of the Streptavidin-Enzyme Conjugate-RTU to each well using a repeater pipette.
- Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **30 minutes** at room temperature ($23 \pm 2^{\circ}\text{C}$).
- Aspirate and wash each strip **5 times** with the Wash Solution (**350 μ L/per well**) using an automatic microplate washer.
- Add **100 μ L** of the TMB chromogen solution to each well using a repeater pipette. Avoid exposure to direct sunlight.
- Incubate the wells, shaking at **600–800 rpm** on an orbital microplate shaker, for **12-14 minutes** at room temperature ($23 \pm 2^{\circ}\text{C}$).
NOTE: Visually monitor the color development to optimize the incubation time.
- Add **100 μ L** of the stopping solution to each well using a repeater pipette. Read the absorbance of the solution in the wells within 20 minutes, using a microplate reader set to **450 nm**.
NOTE: Zero calibrator should be programmed as "Blank" while reading the optical density. If instrument has a wavelength correction, set the instrument to dual wavelength measurement at 450 nm with background wavelength correction at 630 nm.

RESULTS

- Optimum results can be obtained at incubation temperature of (**$23 \pm 2^{\circ}\text{C}$**).
- Calculate the mean OD for each calibrator, Control or Unknown.
- Plot the log of the mean OD readings for each of the Calibrators along the y-axis versus log of the Glucagon concentrations in pg/mL along the x-axis, using a cubic regression curve-fit.
- Determine the Glucagon concentrations of the Controls and unknowns from the calibration curve by matching their mean OD readings with the corresponding Glucagon concentrations.
- Any sample reading higher than the highest Calibrator should be appropriately diluted with another sample with low glucagon concentration or reconstituted Glucagon Sample Diluent and re-assayed.
- Any sample reading lower than the analytical sensitivity should be reported as such.
- Multiply the value by a dilution factor, if required.

LIMITATIONS

The reagents supplied in this kit are optimized to measure Glucagon levels in K_2EDTA and Lithium Heparin plasma samples. If there is evidence of microbial contamination or excessive turbidity in a reagent, discard the vial. For assays employing antibodies, the possibility exists for interference by heterophile antibodies in the samples.⁹

QUALITY CONTROL

- Each laboratory should establish mean values and acceptable ranges to ensure proper performance.
- Glucagon ELISA controls or other commercial controls should fall within established confidence limits.
- The confidence limits for Glucagon controls are printed on the **Calibration card**.
- A full calibration curve, low and high-level controls, should be included in each assay.
- TMB should be colorless. Development of any color may indicate reagent contamination or instability.

REPRESENTATIVE CALIBRATION CURVE DATA

Well Number	Well Contents Calibrators	Mean OD	Conc (pg/mL)
A1, A2	A	0.064 (Blank)	0
B1, B2	B	0.026	7.0
C1, C2	C	0.116	24.0
D1, D2	D	0.273	50.0
E1, E2	E	0.650	97.0
F1, F2	F	1.446	178.0
G1, G2	G	2.868	314.0

CAUTION: The above data must not be employed in lieu of data obtained by the user in the laboratory.

ANALYTICAL CHARACTERISTICS

All concentrations listed are in pg/mL (1pg/mL = 0.3 pmol/L).

Analytical Sensitivity:

The analytical sensitivity in the Glucagon ELISA assay, as calculated by the interpolation of mean plus two standard deviations of 15 replicates of calibrator A (0 pg/mL) and calibrator B (7.0 pg/mL), is 2.4 pg/mL.

Imprecision:

Reproducibility of the Glucagon assay was determined using two kit controls and three pooled samples (n=48 for all). The study included twelve assays with CI, CII and three samples in quadruplets.

Sample ID	Mean Conc. (pg/mL)	Within run		Between run		Total	
		SD	CV	SD	CV	SD	CV
CI	50.5	1.7	3.3%	1.7	3.4%	2.4	4.8%
CII	109.6	5.1	4.7%	0.0	0.0%	5.1	4.7%
1	7.8	1.1	3.4%	0.9	2.8%	1.4	4.4%
	3.1	0.8	1.5%	1.5	2.8%	1.7	3.1%
	91.5	1.5	1.6%	1.8	2.0%	2.3	2.5%

Analytical specificity:

The monoclonal antibody pair used in the assay detects human Glucagon and does not cross-react to other closely related analytes. The antibody pair used in the Glucagon assay detects Goat, Rabbit, Bovine, Equine, Ovine, Porcine, Canine, Mouse, Squirrel Monkey and Rhesus Monkey glucagon.

Cross-reactant	Concentration	% Cross-reactivity
Glucagon (1-27)	100 ng/mL	100%
Glucagon (19-29)	1000 pg/mL	Non-Detectable
Oxyntomodulin (1-37)	1000 ng/mL	Non-Detectable
GLP-1 (7-36)	1000 ng/mL	Non-Detectable
GLP-1 (9-36)	1000 ng/mL	Non-Detectable
GLP-1 (1-36)	1000 pg/mL	Non-Detectable
GLP-2 (1-34)	1000 ng/mL	Non-Detectable
GRPP	1000 ng/mL	Non-Detectable
Glicentin	1000 ng/mL	Non-Detectable
MPGF-2	100 ng/mL	Non-Detectable
Insulin	10 ng/mL	Non-Detectable
C-peptide	10 ng/mL	Non-Detectable
Thyroglobulin	10 ng/mL	Non-Detectable

Note: Cross-Reactivity to Lilly Glucagon, Novo Glucagon, Zealand Glucagon, Xeris Glucagon are available upon request.

Interference:

When potential interferents (Hemoglobin, Biotin, Intralipids and Bilirubin) were added at least at two times their physiological concentration to control

sample, Glucagon concentrations were within ±10% of the control as represented in the table below.

Interferent	Interferent Dose	Sample (pg/mL)	Dosed Sample (pg/mL)	% Difference
Hemoglobin	1 mg/mL	78.38	80.31	2.5
	0.5 mg/mL	80.00	78.09	-2.4
	1 mg/mL	217.16	213.85	-1.5
	0.5 mg/mL	227.17	231.64	2.0
Biotin	1200 ng/mL	71.39	75.97	6.4
	600 ng/mL	78.25	77.35	-1.1
	1200 ng/mL	204.48	194.55	-4.9
Intralipids	20 mg/mL	69.13	67.54	-2.3
	10 mg/mL	74.63	73.80	-1.1
	20 mg/mL	207.62	204.32	-1.6
Bilirubin	10 mg/mL	203.80	219.81	7.9
	0.66 mg/mL	43.06	41.4	-1.4
	0.2 mg/mL	68.23	66.43	-3.3
	0.66 mg/mL	122.85	118.92	-3.2
	0.2 mg/mL	189.47	182.93	-3.4

Dilution Recovery:

Multiple dilutions of Calibrator F and three samples containing various Glucagon levels were diluted with SPD-157. The % recovery of individual samples is represented in the following table.

High reading sample should be diluted with low reading sample. Sample diluent (SPD-157) provided in the kit can be used to dilute high reading samples up to 35 pg/mL.

Sample ID	Dilution Factor	Expected Conc (pg/mL)	Observed Conc (pg/mL)	%Recovery
Calibrator F	Neat	300.00	NA	NA
	2	150.00	157.94	105%
	4	75.00	78.21	104%
	8	37.50	42.47	113%
S1	Neat	68.07	NA	NA
	2	34.04	29.73	87%
S2	Neat	64.56	NA	NA
	2	32.28	31.25	97%
S3	Neat	84.40	NA	NA
	2	42.20	35.20	83%
	4	21.10	22.78	108%

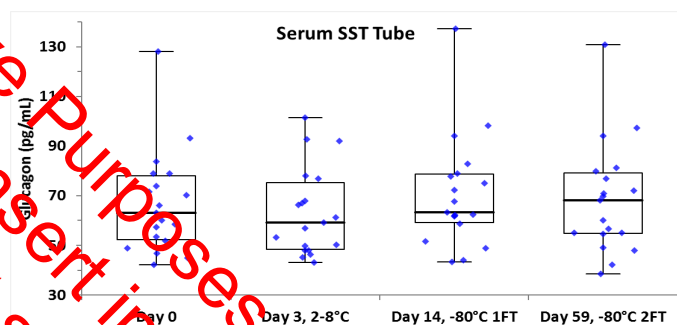
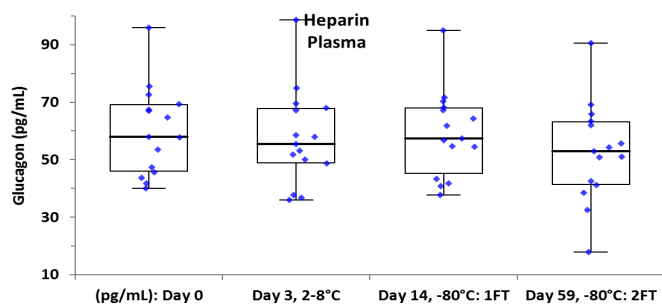
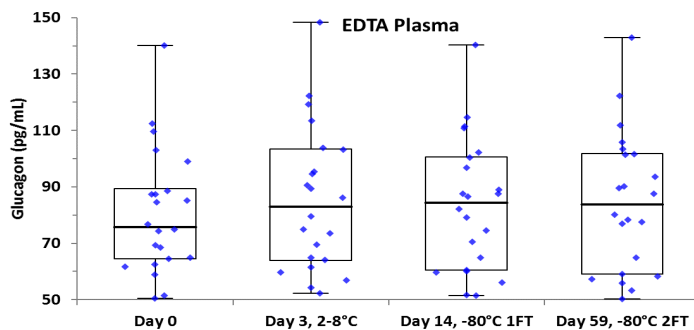
Spike Recovery:

Two samples were used for sample-to-sample spike. Low reading K₂EDTA Glucagon samples was spiked with high reading K₂EDTA sample.

Sample ID	Endogenous Value in pg/mL	Expected in pg/mL	Observed in pg/mL	%Recovery
1	5.1330	15.55	17.39	112%
		36.41	30.87	85%
2	0.0010	8.83	10.10	114%
		26.47	28.44	107%

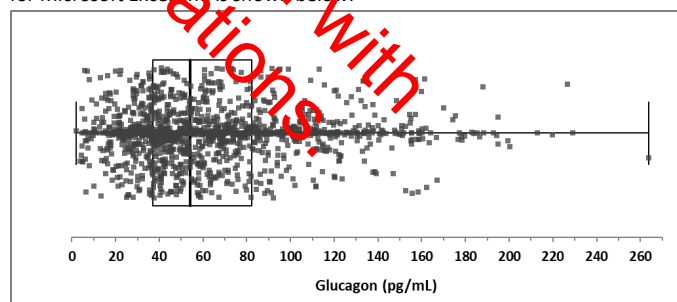
Sample Stability

Twenty-five matched Serum, Lithium Heparin and dipotassium EDTA plasma were collected in BD tubes, processed, and run fresh aliquoted and stored at 2-8 and -80°C. The results are plotted below.



Expected Values:

Expected Glucagon concentration in undifferentiated population (diabetic and non-diabetic) was calculated by evaluating 1533 samples in Ansh Labs Glucagon ELISA. The frequency distribution was calculated using Analyse-It® for Microsoft Excel and is shown below.



n	Glucagon (pg/mL)			
	Mean	Median	Range	95% CI
1533	63.9	54.2	2.1 - 263.8	13.1 - 158.7
Quantile		Glucagon (pg/mL)		
0.1		25.2		
0.2		34.1		
0.3		39.6		
0.4		45.9		

0.5	54.2
0.6	64.4
0.7	75.1
0.8	90.8
0.9	118.1

NOTE: It is recommended that each laboratory should determine the reference range(s) for its own patient population. The results of this assay should be used in conjunction with other relevant and applicable clinical information.

REFERENCES

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FOR EXPORT ONLY

Symbols used with Ansh Labs Assays

Symbol	English	Deutsch	Français	Español	Italiano
	European Conformity	CE-Konformitätskennzeichnung	Conformité aux normes européennes	Conformidad europea	Conformità europea
	Caution	Vorsicht	mise en garde	precaución	attenzione
	Consult instructions for use	Gebrauchsanweisung beachten	Consulter les instructions d'utilisation	Consulte las instrucciones de uso	Consultare le istruzioni per l'uso
	In vitro diagnostic device	In-vitro-Diagnostikum	Usage Diagnostic in vitro	Para uso Diagnóstico in vitro	Per uso Diagnostica in vitro
	Catalogue number	Katalog-Nr.	Numéro de catalogue	Número de catálogo	Numero di Catalogo
	Lot No. / Batch code	Chargen-Nr.	Numéro de lot	Número de lote	Numero di lotto
	Storage Temperature	Lagerungstemperatur	Température de conservation	Temperatura de conservación	Temperatura di conservazione
	Expiration Date	Mindesthaltbarkeitsdatum	Date limite d'utilisation	Fecha de caducidad	Data di scadenza
	Date of Manufacture	Herstellungsdatum	date de fabrication	fecha de manufactura	Data di produzione
	Legal Manufacturer	Hersteller	Fabricant	Fabricante	Fabbricante
Content	Content	Inhalt	Conditionnement	Contenido	Contenuto
Volume/No.	Volume / No.	Volumen/Anzahl	Volume/Quantité	Volumen/Número	Volume/Quantità
	96-well plate	Platte mit 96 Vertiefungen	Plaque à 96 puits	placa de 96 pocillos	piastra a 96 pozzetti

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the product for exact specifications.