Proglucagon ELISA

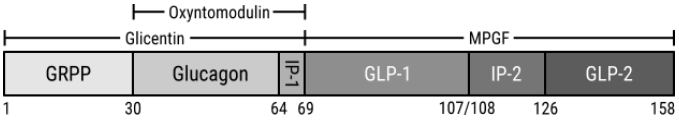
RUO

AL-1019-r

INTENDED USE

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

SUMMARY AND EXPLANATION



Proglucagon, a 158 amino acid polypeptide, is synthesized in the pancreatic alpha cells and the intestinal L-cells[1]. Its post-translational processing in the pancreas leads to GRPP, Glucagon, IP-1 and MPGF. While in the intestines, the post-translational process leads to Glicentin, Oxyntomodulin, GLP-1, IP-2 and GLP-2.[2] Analogs and agonists of these proglucagon factors are used in the treatment and management of Type-1 and Type-2 diabetes and prevention of cardiovascular complications associated with diabetes.

PRINCIPLE OF THE TEST

The Proglucagon ELISA is a quantitative two-step sandwich type immunoassay. In the first step Calibrators, Controls and unknown samples are added to Proglucagon antibody coated microtiter wells and incubated with biotinylated Proglucagon 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 Proglucagon in the samples and calibrators.

MATERIALS SUPPLIED

CAL-1019A - CAL-1019F Proglucagon Calibrator A thru F (Lyophilized)

Six vials, labeled A-F, containing concentration of approximately 0-1000 pg/mL Proglucagon in protein based buffer and Pro-Clean 400. Refer to **calibrator card** for exact concentrations. Store unopened at 2 to 8°C until the expiration date. Reconstitute calibrators A-F with 1 mL deionized water. Solubilize, mix well, and use after reconstitution.

CTR-1019-I & CTR-1019-II Proglucagon Controls I & II (Lyophilized)

Two vials, labeled Levels I and II containing low and high Proglucagon concentrations in heat treated serum with non-mercury preservative. Refer to **calibrator 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.

PLT-1019 Proglucagon Coated Microtitration Strips

One strip-holder, containing 12 strips and 96 microtitration wells with Proglucagon 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.

BCR-1019 Proglucagon Biotin Conjugate Ready-To-Use (RTU)

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

SAR-1019 Proglucagon 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 *research use only*.

The following precautions should be observed:

a) Follow good laboratory practice.
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, 2007.[6]

WARNING: Potential Chemical Hazard

Some reagents in this kit contain Pro-Clean 400 and Sodium azide[7] as a preservative. Pro-Clean 400 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, Proglucagon 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 Proglucagon 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 Proglucagon 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 Proglucagon 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 ± 2°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. **Proglucagon Calibrators A–F and Proglucagon Controls I & II:** Tap and reconstitute Proglucagon Calibrators A-F with 1.0 mL deionized water. Solubilize for 10 minutes, mix well and use after reconstitution.
2. **Wash Solution:** Dilute wash concentrate 25-fold with deionized water. The wash solution is stable for one month at room temperature (23 ± 2°C) when stored in a tightly sealed bottle.
3. **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 ± 2°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 Proglucagon Cal-1019A prior to assay.*

1. Reconstitute Proglucagon Calibrators A-F and Proglucagon Controls I & II each with **1.0 mL** deionized water. Solubilize for **10 minutes**, Mix well.
2. Label the microtitration strips to be used.
3. Pipette **25 µL** of the Calibrator, Controls and Unknowns to the appropriate wells.
4. Add **100 µL** of the Antibody-Biotin Conjugate RTU to each well using a repeater pipette.
5. Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **2 hours** at room temperature (23 ± 2°C).
6. Aspirate and wash each strip **5 times** with Washing Solution (**350 µL/per well**) using an automatic microplate washer.
7. Add **100 µL** of the Streptavidin-Enzyme Conjugate-RTU to each well using a repeater pipette.
8. Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **30 minutes** at room temperature (23 ± 2°C).
9. Aspirate and wash each strip **5 times** with the Wash Solution (**350 µL/per well**) using an automatic microplate washer.
10. Add **100 µL** of the TMB chromogen solution to each well using a repeater pipette. Avoid exposure to direct sunlight.
11. Incubate the wells, shaking at **600–800 rpm** on an orbital microplate shaker, for **10-12 minutes** at room temperature (23 ± 2°C).

    NOTE: *Visually monitor the color development to optimize the incubation time*.
12. Add **100 µL** 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

1. Optimum results can be obtained at incubation temperature of (**23 ± 2°C**).
2. Calculate the mean OD for each Calibrator, Control, or Unknown.
3. Plot the log of the mean OD readings for each of the Calibrators along the y-axis versus log of the Proglucagon concentrations in pg/mL along the x-axis, using a cubic regression curve-fit.

4. Determine the Proglucagon concentrations of the Controls and unknowns from the calibration curve by matching their mean OD readings with the corresponding Proglucagon concentrations.
5. Any sample reading higher than the highest Calibrator should be appropriately diluted with another sample with low proglucagon concentration or reconstituted Proglucagon Cal-1019A and re-assayed.
6. Any sample reading lower than the analytical sensitivity should be reported as such.
7. Multiply the value by a dilution factor, if required.

LIMITATIONS

The reagents supplied in this kit are optimized to measure Proglucagon 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 assure proper performance.
- Proglucagon ELISA controls or other commercial controls should fall within established confidence limits.
- The confidence limits for Proglucagon controls are printed on the **Calibrator 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	0.043 (**Blank**)	0
B1, B2	B	0.078	6.48
C1, C2	C	0.136	17.8
D1, D2	D	0.391	66.2
E1, E2	E	1.366	252.6
F1, F2	F	3.718	913.0

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

ANALYTICAL CHARACTERISTICS

Analytical Sensitivity:

The analytical sensitivity in the Proglucagon ELISA assay, as calculated by the interpolation of mean plus two standard deviations of 16 replicates of calibrator A (0 pg/mL) and calibrator B (6.48 pg/mL), is 1.14 pg/mL.

Imprecision:

Reproducibility of the Proglucagon ELISA assay was determined in a study using three serum pools. The study included a total of 10 assays, 3 replicates of each per assay (n=30). Representative data are presented in the following table.

Sample	Mean Conc.	Within run		Between run		Total	
	pg/mL	SD	%CV	SD	%CV	SD	%CV
1	17.3	0.7	4.2%	0.7	4.2%	1.0	5.9%
2	22.0	0.8	3.7%	0.9	4.1%	1.2	5.5%
3	81.8	2.5	3.1%	4.0	4.8%	4.7	5.7%

Analytical Specificity:

The antibody pair used in the Proglucagon ELISA measures proglucagon and does not detect other similar molecules. The assay does not detect Glucagon, GLP-1, GLP-2, Oxyntomodulin, GRPP, MPGF-1, MPGF-2, Insulin, C-peptide, and Thyroglobulin.

Cross-Reactant	Concentration	% Cross-reactivity
Glucagon	100 ng/mL	ND
GLP-1	100 ng/mL	ND
GLP-2	100 ng/mL	ND
Oxyntomodulin	50 ng/mL	ND
GRPP	100 ng/mL	ND
MPGF-1	100 ng/mL	ND
MPGF-2	100 ng/mL	ND
Insulin	100 ng/mL	ND
C-Peptide	100 ng/mL	ND
Thyroglobulin	100 ng/mL	ND

Linearity:

Proglucagon Antigen at a high concentration was diluted with Calibrator A/sample diluent. The % recovery is represented in the following table.

Sample ID	Dilution factor (1 in X)	Expected Value in pg/mL	Observed Value in pg/mL	% Recovery	Average %Recovery
Proglucagon Antigen	**NEAT**	758	Neat	**N/A**	**98%**
	2	379	373.6	**99%**	
	4	189.5	186.7	**99%**	
	8	94.8	90.4	**95%**	
	16	47.4	45.3	**96%**	
	32	23.7	23.9	**101%**	

Recovery:

Plasma samples at a low and high Proglucagon levels were mixed at different levels and the % recovery was calculated.

Sample	Endogenous Conc. (pg/mL)	Expected Conc. (pg/mL)	Observed Conc. (pg/mL)	%Recovery
S1	20.0	22.2	21.6	96%
		24.3	22.6	
		26.4	25.8	
S2	22.9	24.9	24.6	98%
		26.9	26.3	
		28.9	28.1	
S3	19.0	21.2	20.9	96%
		23.4	21.6	
		25.6	24.5	

REFERENCES

1. JACQUES PHILIPPE, SVETLANA MOJSOV, DANIEL J. DRUCKER, JOEL F. HABENER, Proglucagon Processing in a Rat Islet Cell Line Resembles Phenotype of Intestine Rather than Pancreas, *Endocrinology*, Volume 119, Issue 6, 1 December 1986, Pages 2833–2839
2. Raffort, J., Lareyre, F., Massalou, D., Fénichel, P., Panaïa-Ferrari, P., & Chinetti, G. (2017). Insights on glicentin, a promising peptide of the proglucagon family. Biochemia medica, 27(2), 308–324.
3. Sandoval DA and D'Alessio DA. (2015) Physiology of Proglucagon Peptides: Role of Glucagon and GLP-1 in Health and Disease. Physiol Rev 95:513-548.
4. Bagger JI et al (2011) Glucagon antagonism as a potential therapeutic target in type 2 diabetes. Diabetes, Obesity and Metabolism 13:965-971.

5. Holst J et al (2004) Role of incretin hormones in the regulation of insulin secretion in diabetic and nondiabetic humans. Am J Physiol Endocrinol Metab 287: E199-206.
6. Holst J (2010) Glucagon and Glucagon-Like Peptides 1 and 2. Results Probl Cell Differ 50:121-135.
7. Young A (2005) Inhibition of Glucagon Secretion. Advances in Pharmacology 52:151-171.
8. HHS Publication, 5th ed., 2007. Biosafety in Microbiological and Biomedical Laboratories. Available http://www.cdc.gov/biosafety/publications/bmbl5/BMBL5
9. DHHS (NIOSH) Publication No. 78–127, August 1976. Current Intelligence Bulletin 13 - Explosive Azide Hazard. Available http:// www.cdc.gov/niosh.
10. Approved Guideline – Procedures for the Handling and Processing of Blood Specimens, H18-A3. 2004. Clinical and Laboratory Standards Institute.
11. Kricka L. Interferences in immunoassays – still a threat. Clin Chem 2000; 46: 1037–1038.

RESEARCH USE ONLY

This assay is intended for research use only.

Manufactured by:
Ansh Labs
445 Medical Center Blvd.
Webster, TX 77598-4217, U.S.A.