C-PEPTIDE ELISA

RUO

AL-151

INTENDED USE

The C-Peptide of Insulin Enzyme-Linked Immunosorbent (ELISA) Kit provides materials for the quantitative measurement of C-peptide of insulin in human serum. The kit is intended for **research use only**.

SUMMARY AND EXPLANATION

Insulin is a member of a family of structurally-related regulatory proteins; other proteins in this group include the insulin-like growth factors and relaxin. Insulin is produced by the β-cells of the pancreatic islets and is initially synthesized as a 12 kDa pre-prohormone, which undergoes intracellular processing to a 9 kDa, 86-amino acid prohormone and subsequent packaging in storage granules. Within these granules, disulfide bonds are formed between the A and B chains of the insulin molecule and the C-peptide region is cleaved, resulting in the 51-amino acid, 6 kDa mature insulin molecule. Upon stimulation, the islet cells release equimolar amounts of insulin and C-peptide, and small amounts of proinsulin and other intermediates (<5% of normal total insulin secretion)[1].

Insulin is the most important hormone of the fed-state, and is the only physiologic hormone which significantly lowers blood glucose levels. In response to a number of substrates and other stimuli, including glucose and amino acids, insulin is secreted into the hepatic portal circulation[1,2]. Fifty-percent of the insulin is removed on first-pass through the liver, the remainder enters the general circulation and is carried to other target tissues. Insulin then binds to specific cell-surface receptors[3] and, through incompletely defined mechanisms, facilitates substrate uptake and intracellular utilization, resulting in net increases in intracellular lipid, protein and glycogen[1-4]. In addition to its role in peripheral metabolism, insulin may influence central regulation of energy balance[5]. Insulin is rapidly cleared both by liver uptake, tissue utilization and renal clearance ($T_{1/2}$ of about 4 mins), and circulating insulin levels are very low during fasting. In contrast, C-peptide of insulin does not undergo significant liver or extra-renal metabolism and, therefore, has a much longer circulating half-life (~30 min).[1]

Basal- and glucose-stimulated circulating insulin concentrations are relatively stable during infancy and childhood, and increase during puberty due to decreased insulin sensitivity [6]. Insulin concentrations tend to be higher in obese individuals, particularly those with an increased proportion of visceral (abdominal) fat[7]. Glucose counter-regulatory hormones, such as glucagon, glucocorticoids, growth hormone and epinephrine, decrease insulin sensitivity and action; insulin levels may increase during exogenous administration of these substances. [1,2]

Measurement of circulating insulin concentrations may be useful in the clinical evaluation of several conditions. Elevated serum insulin levels in the presence of low glucose concentrations may be indicative of pathologic hyperinsulinism, e.g. nesidioblastosis and islet-cell tumor [8]. Elevated serum fasting insulin levels with normal or elevated glucose concentrations, and exaggerated insulin and glucose response to exogenous glucose administration are characteristic of the insulin-resistant forms of glucose intolerance and diabetes mellitus and other insulin resistant conditions.[7, 9], [10] High circulating insulin concentrations may be involved in the pathogenesis of hypertension and cardiovascular disease[10,11]. Conversely, low insulin concentrations in the presence of hyperglycemia suggests insulin-deficiency, e.g. insulin-dependent or Type I diabetes mellitus.

Although the C-peptide of insulin is biologically inactive, it has a longer circulating half-life than insulin and undergoes relatively minimal hepatic metabolism. In addition, C-peptide of insulin assays may be analytically more sensitive than insulin assays. Because of these factors, measurement of C-peptide of insulin may be useful in evaluating insulin secretion in a variety of clinical conditions.[12-14]

PRINCIPLE OF THE TEST

C-Peptide of Insulin ELISA is an enzymatically amplified "one-step" sandwich-type immunoassay. In the assay, Standards, Controls and unknown serum samples are incubated with anti-C-peptide of insulin antibody in microtitration wells which have been coated with another anti-C-peptide of insulin antibody. After incubation and washing, the wells are incubated with the substrate tetramethylbenzidine (TMB). An acidic stopping solution is then added and the degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurement at 450 and 620 nm.

The absorbance measured is directly proportional to the concentration of C-peptide of insulin present. A set of C-Peptide Standards is used to plot a standard curve of absorbance versus C-peptide of insulin concentration from which the C-peptide of insulin concentrations in the unknowns can be calculated.

MATERIALS SUPPLIED

CAL-151A C-Peptide Calibrator A /Sample Diluent

One bottle, 8 mL, labeled A, containing 0 ng/mL C-peptide of Insulin in a protein based buffer containing Pro-Clean 400. Store unopened at 2 to 8°C until the expiration date. Stored at 2 to 8°C until expiration date.

CAL-151B - CAL-151F C-Peptide Calibrators B thru F (Lyophilized)

Five vials, labeled B-F, containing concentrations of approximately 0.2-10.9 ng/mL C-Peptide of Insulin in a protein based buffer containing Pro-Clean 400. Refer to **calibration card** for exact concentrations. Store unopened at 2 to 8°C until the expiration date. Reconstitute calibrators B-E with **1.0 mL** deionized water. Solubilize for **10 minutes**, mix well and use after reconstitution. For longer storage after reconstitution, aliquot and freeze at -20°C or colder for up to one year.

***NOTE**: The calibrators are traceable to World Health Organization Human C-Peptide preparation NIBSC code 13/146, version 3.0.*

CTR-151-I & CTR-151-II C-Peptide Controls I & II (Lyophilized)

Two vials, labeled Levels I and II containing low and high C-Peptide of Insulin concentrations in a protein based buffer containing Pro-Clean 400. 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 **1.0 mL** deionized water. Solubilize for **10 minutes**, mix well and use after reconstitution. For longer storage after reconstitution, aliquot and freeze at -20°C or colder for up to one year.

PLT-151 C-Peptide Antibody Coated Microtitration Strips

One stripholder, containing 12 strips and 96 microtitration wells with anti-C-Peptide of Insulin 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.

ECR-151 C-Peptide Antibody Enzyme Conjugate—Ready-to-Use (RTU)

One bottle, 12 mL, containing a protein based buffer with Pro-Clean 400. Store at 2-8°C until expiration date.

TMB-100 TMB Chromogen Solution

One bottle, 11 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, 11 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, 405 nm 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.

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

Samples and blood-derived products may be routinely processed with minimum risk using the procedure described. However, handle these products as potentially infectious according to universal precautions and good clinical laboratory practices, regardless of their origin, treatment or prior certification.[15] Use an appropriate disinfectant for decontamination. Store and dispose of these materials and their containers in accordance with local regulations and guidelines.

WARNING: Potential Chemical Hazard

Some reagents in this kit contain Pro-Clean 400 and Sodium azide[16] as a preservative. Pro-Clean 400 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) Serum is the recommended sample type.
b) Fasting serum should be used and the usual precautions for venipuncture should be observed. Store serum at -70°C. Samples should be stable for at least 30 days when stored frozen at -70°C [15]. Do not use hemolyzed or lipemic specimens.
c) 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.[17]

PROCEDURAL NOTES

1. A thorough understanding of this package insert is necessary for successful use of the C-Peptide 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 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. **C-Peptide Calibrators B-F**: Tap and reconstitute C-Peptide Calibrators B-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 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 and mix thoroughly by gentle inversion before use. Calibrators, controls, and unknowns should be assayed in duplicate.

NOTE: *All serum samples reading higher than the highest calibrator should be mixed and diluted in the 0 ng/mL Calibrator A/Sample Diluent prior to assay.*

1. Reconstitute C-Peptide Calibrators B-F and C-Peptide 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 **20 µL** of the Calibrators, Controls and Unknowns to the appropriate wells.
4. Add **25 µL** calibrator A/sample diluent to each well using a repeater pipette.
5. Add **100 µL** C-Peptide Antibody Enzyme Conjugate solution 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 **1 hour** at room temperature.
7. Aspirate and wash each strip **5 times** with Washing Solution **(350 µL/per well**) using an automatic microplate washer.
8. Add **100 µL** of the TMB chromogen solution to each well using a precision pipette. Avoid exposure to direct sunlight.
9. Incubate the wells, shaking at **600–800 rpm** on an orbital microplate shaker, for **10-12 min** at room temperature.
   ***NOTE**: Visually monitor the color development to optimize the incubation time.*
10. Add **100 µL** of the stopping solution to each well using a precision pipette. Read the absorbance of the solution in the wells within **20 minutes**, using a microplate reader set to **450 nm.**
    ***NOTE**: While reading the absorbance of the microtitration well, it is necessary to program the zero calibrator as a "Blank".*

RESULTS

***NOTE:** The results in this package insert were calculated by plotting the data on a log vs. log scale using a cubic regression curve-fit. Other data reduction methods may give slightly different results.*

1. Calculate the mean OD for each calibrator, Control, or Unknown.
2. Plot the log of the mean OD readings for each of the Calibrators along the y-axis versus log of the C-Peptide of Insulin concentrations in ng/mL along the x-axis, using a cubic regression curve-fit.
3. Determine the C-Peptide of Insulin concentrations of the Controls and unknowns from the calibration curve by matching their mean OD readings with the corresponding C-Peptide of Insulin concentrations.
4. Any sample reading higher than the highest Calibrator should be appropriately diluted with the 0 ng/mL (CAL A) and re-assayed.
5. Any sample reading lower than the analytical sensitivity should be reported as such.
6. Multiply the value by a dilution factor, if required.

LIMITATIONS

The reagents supplied in this kit are optimized to measure C-Peptide of Insulin levels in human serum. 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.[18]

The C-Peptide of Insulin ELISA results should be interpreted with respect to the total clinical presentation of the patient, including: symptoms, clinical history, data from additional tests, and other appropriate patient examination information.

QUALITY CONTROL

- Each laboratory should establish mean values and acceptable ranges to assure proper performance.
- C-Peptide of Insulin ELISA controls or other commercial controls should fall within established confidence limits.
- The confidence limits for C-Peptide of Insulin 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	Mean OD	Conc. (ng/mL)
	Calibrators		
A1, A2	A	0.002 (**Blank**)	0
B1, B2	B	0.016	0.2
C1, C2	C	0.09	0.77
D1, D2	D	0.406	2.33
E1, E2	E	1.601	6.3
F1, F2	F	3.346	10.9

***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 ng/mL.

Imprecision:

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

Sample	Mean Conc.	Within run		Between run		Total	
ID	**ng/mL**	**SD**	**CV**	**SD**	**CV**	**SD**	**CV**
CI	1.054	0.031	2.96%	0.000	0.00%	0.031	2.96%
CII	4.441	0.065	1.46%	0.061	1.37%	0.089	2.00%
1	0.411	0.011	2.69%	0.004	0.88%	0.012	2.83%
2	1.488	0.032	2.14%	0.012	0.79%	0.034	2.28%
3	3.698	0.085	2.29%	0.066	1.78%	0.107	2.90%

Analytical Sensitivity:

The analytical sensitivity in the C-Peptide ELISA assay, as calculated by the interpolation of mean plus two standard deviations of 8 replicates of calibrator A (0 ng/mL) and calibrator B (0.2 ng/mL), is 0.018 ng/mL.

Analytical Specificity:

The monoclonal antibody pair used in the assay detects human, bovine, canine, rabbit and goat C-peptide and does not cross-react to other closely related analytes at 1000 ng/mL as shown in the following table.

Cross-Reactant	Cross-reactivity
Oxyntomodulin (1-37)	ND
Glucagon (1-29)	ND
GLP-1 (7-36)	ND
GLP-1 (9-36)	ND
GLP-2 (1-34)	ND
GRPP	ND
Insulin	ND
IGF-I	ND
IGF-II	ND

Recovery:

Known amounts of C-Peptide were added to four serum samples containing different levels of endogenous C-Peptide. The concentration of C-Peptide was determined before and after the addition of exogenous C-Peptide and the percent recovery was calculated.

Sample	Endogenous Conc. (ng/mL)	Expected Concentration (ng/mL)	Observed Concentration (ng/mL)	%Recovery
1	1.4560	1.928	2.001	104%
		2.400	2.499	104%
		2.873	3.016	105%
2	0.9130	1.412	1.510	107%
		1.912	2.049	107%
		2.411	2.621	109%
3	0.7750	1.281	1.360	106%
		1.788	1.951	109%
		2.294	2.460	107%
4	0.1790	0.715	0.847	118%
		1.251	1.433	115%
		1.787	1.955	109%

Linearity:

Two serum samples containing various C-Peptide levels were serially diluted in Calibrator A/Sample Diluent. The percent (%) recovery on individual samples is represented in the following table.

Sample	Dilution Factor	Expected Conc. (ng/mL)	Observed Conc. (ng/mL)	% Recovery
1	Neat	8.19	Neat	NA
	1:02	4.1	3.71	91%
	1:04	2.05	1.74	85%
	1:08	1.02	0.89	87%
	1:16	0.51	0.47	92%
	1:32	0.26	0.24	94%
2	Neat	10.03	Neat	NA
	1:02	5.02	4.69	93%
	1:04	2.51	2.33	93%
	1:08	1.25	1.24	99%
	1:16	0.63	0.67	106%
	1:32	0.31	0.33	106%

Interference:

When potential interferents (hemoglobin, triglycerides, bilirubin and biotin) were added at least at two times their physiological concentration to control sample, C-peptide concentration were within ± 10% of the control as represented in the following table.

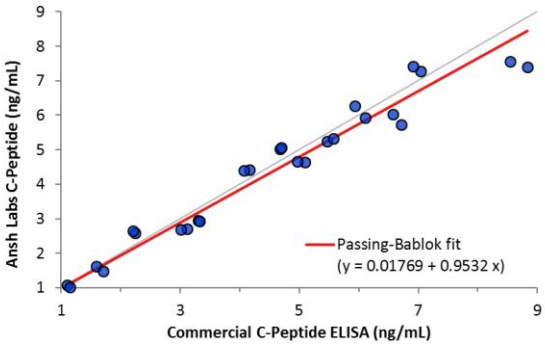
Interferents	Analyte Conc. (mg/mL)	Unspiked Sample Value (ng/mL)	Spiked Sample Value (ng/mL)	% Difference
Hemoglobin	1.35	1.377	1.381	0.290
		2.37	2.459	3.755
		6.958	7.004	0.661
Triglycerides	10.00	1.333	1.324	-0.675
		2.233	2.275	1.881
		6.475	6.729	3.923
Bilirubin	0.60	1.373	1.362	-0.801
		2.255	2.2	-2.439
		6.855	6.724	-1.911
Biotin	6 X10^{-5}	1.467	1.435	-2.181
		2.465	2.494	1.176
		7.38	7.444	0.867

Method Comparison

Eleven serum specimens in the range of 1.1-8.8ng/mL were compared to commercial C-Peptide ELISA assay (Method A).

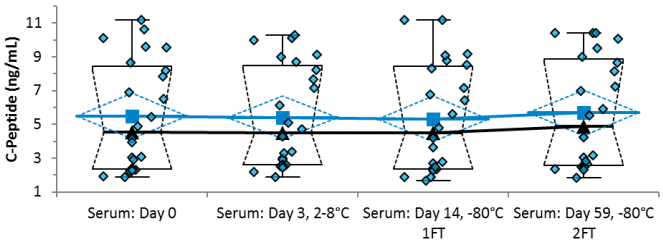
Passing Bablok analysis of the results yielded the following Regression:

Ansh Labs (AL-151) = 0.95 (Method A) + 0.018 ng/mL, (r=0.976)



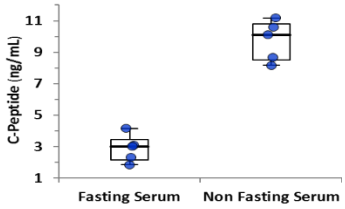
Sample Study

Twenty-four serum specimens in the range of 1.8-11.1ng/mL were aliquoted and stored at different temperatures for indicated number of days and compared to freshly drawn serum samples in C-Peptide ELISA assay (AL-151).



	N	Mean	95% CI
Serum: Day 0	24	5.4990	4.1400 to 6.8581
Serum: Day 3, 2-8°C	24	5.4064	4.1385 to 6.6743
Serum: Day 14, -80°C	24	5.3179	3.9706 to 6.6651
Serum: Day 59, -80°C	24	5.6875	4.3431 to 7.0320

Five matched fasting and non-fasting serum specimens were compared in C-Peptide ELISA assay (AL-151) and the results are shown in the following table



ID	Median (ng/mL)
Fasting Serum	3.02
Non-Fasting Serum	10.1

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