

Total IGFBP-4 ELISA

RUO

AL-126

INTENDED USE

The Total IGFBP-4 enzyme linked immunosorbent assay (ELISA) kit provides materials for the quantitative measurement of IGFBP-4 in serum. This kit is intended for laboratory **research use only** and is not for use in diagnostic or therapeutic procedures.

SUMMARY AND EXPLANATION

Insulin-like growth factor-binding protein-4 is a member of the insulin-like growth factor binding protein (IGFBP) family and encodes a protein with an IGFBP domain and a thyroglobulin type-I domain. The cDNA for human IGFBP-4 encodes a 258-residue protein that is processed, by removal of the signal sequence, to a mature protein of 237 residues (25.6 kDa) with a single asparagine-linked glycosylation site¹. Although various cell types when in culture secrete both glycosylated (28-29 kDa) and nonglycosylated (24-25 kDa) forms of IGFBP-4, the nonglycosylated is typically the most abundant in normal human blood^{2,3}.

IGFBP-4 is unique among the six IGFBPs in having two extra cysteine residues in the variable L-domain and may be responsible for the distinctive biological functions of IGFBP-4 (4). Although the exact functional role for serum IGFBP-4 is not absolutely clear, *in vitro* studies have shown that IGFBP-4 inhibits IGF activity in bone cells and other cell types. IGFBP-4 has been reported to inhibit IGF-I- and IGF-II-induced cell proliferation of embryonic chick calvaria cells and MC3T3-E1 mouse osteoblasts^{5,6}, IGF-I- and IGF-II stimulated DNA synthesis in a variety of cell types.⁽³⁾

Proteolysis is a major regulatory mechanism of IGFBP-4 functions. An IGF-dependent IGFBP-4-specific protease was first reported in the media conditioned by both human and sheep dermal fibroblasts. This protease was later identified as pregnancy-associated plasma protein-A (PAPP-A). It was shown that recombinant PAPP-A is an active protease able to cleave IGFBP-4 at a single site, between M135/K136. IGFBP-4 cleavage by PAPP-A is possible only in case when IGFBP is complexed with IGF. PAPP-A also cleaves IGFBP-5 between S143/K144, but in this case the presence of IGF is not required.

Several studies have shown that concentration of PAPP-A in blood of patients with acute coronary syndrome (ACS) is higher than in blood of patients with stable coronary artery disease or control subjects. PAPP-A has been suggested as a marker of cardiovascular diseases associated with coronary artery blood clotting, such as unstable angina and myocardial infarction (MI)⁷⁻¹⁴. It was hypothesized that in atherosclerotic plaques PAPP-A expressed by activated smooth muscles cells could function as an active enzyme cleaving IGFBP-4 complexed with IGF, thus enhancing IGF bioavailability. The IGF system might contribute to the atherosclerotic plaque development, destabilization, and rupture leading to acute coronary events¹⁵. It was shown that IGFBP-4 is expressed by different cells of tumor origin, such as lung adenocarcinoma, non-small-cell lung cancer, breast cancer, colon carcinoma, follicular thyroid carcinoma, gastric cancer, glioma, hepatoma, myeloma, neuroblastoma, osteosarcoma and prostate cancer. *In vitro* and *in vivo* studies suggest that IGFBP-4 plays an important role in the growth regulation of a variety of tumors, possibly by inhibiting autocrine IGF actions. Regulation of IGF bioavailability may play a crucial role in tumor growth and development¹³.

The measurements of IGFBP-4 along with PAPP-A enzyme activity could be of higher clinical value than just PAPP-A measurements alone since PAPP-A concentration in blood is affected by heparin injections. The concentration of PAPP-A, total IGFBP-4 and intact IGFBP-4 in biological fluid can be measured accurately using immunoassay methods (picoPAPP-A ELISA, AL-101; Total IGFBP-4 ELISA, AL-126; and Intact IGFBP-4 ELISA, AL-128 respectively). The ratio of total to Intact IGFBP-4 concentration measured in individual subjects over time may help normalize the IGFBP-4 variability between subjects and also increase the detection of increased PAPP-A activity in MI subjects. The immunoassay methods designed for the measurement of total and Intact IGFBP-4 in patient samples could be of practical value for the diagnosis or prediction of various pathologies including ACS and cancer.

PRINCIPLE OF THE TEST

The Total IGFBP-4 is a quantitative three-step sandwich type immunoassay. In the first step Calibrators, Controls and unknown samples are added to IGFBP-4 antibody coated micro titer wells and incubated. After the first incubation, and washing, the wells are incubated with biotinylated IGFBP-4 antibody solution. After the second incubation and washing, the wells are incubated with streptavidin horseradish peroxidase conjugate (SHRP) solution. After the third incubation and washing step, the wells are incubated with substrate solution (TMB) followed by an acidic stopping solution. 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 Total IGFBP-4 in the samples and calibrators.

MATERIALS SUPPLIED

CAL-126A Total IGFBP-4 Calibrator A

One bottle, 2.5 mL, labeled Total IGFBP-4 Cal A, containing 0 ng/mL IGFBP-4 in protein based buffer and Pro-Clean 400. Store unopened at 2-8°C until the expiration date.

CAL-126B - CAL-126F Total IGFBP-4 Calibrators B-F (Lyophilized)

Five vials, labeled B-F, containing concentrations of approximately 50-800 ng/mL IGFBP-4 in protein based buffer and Pro-Clean 400. Refer to **calibration card** for exact concentrations. Store unopened at 2 to 8°C until the expiration date. Reconstitute calibrators B-F with 0.5 mL deionized water. Solubilize, Mix well and use after reconstitution. Aliquot and freeze in plastic vials immediately for multiple assay use and discard after the run. Avoid repeated freeze thaws. The IGFBP-4 concentration in the IGFBP-4 calibrators is traceable to the manufacturer's working calibrators. Values assigned by other methodologies may be different. Such differences, if present, may be caused by inter-method bias.

CTR-126-I & CTR-126-II Total IGFBP-4 Controls I & II

Two vials, labeled Levels I and II containing low and high IGFBP-4 concentrations in protein based buffer and Pro-Clean 400. Refer to **calibration card** for exact concentrations. Store unopened at 2 to 8°C until the expiration date. Reconstitute control Levels I and II with 0.5 mL deionized water. Solubilize, Mix well and use after reconstitution. Aliquot and Freeze immediately in plastic vials for multiple assay use and discard after run. Avoid repeated freeze thaws.

PLT-126 IGFBP-4 Coated Microtitration strips

One strip holder, containing 12 strips and 96 microtitration wells with anti IGFBP-4 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.

ASB-126 IGFBP-4 Assay Buffer

One bottle, 22 mL, containing a protein-based (BSA)-buffer with a non-mercury preservative. Store at 2-8°C until expiration date.

BCC-126 Total IGFBP-4 Biotin Conjugate Concentrate

One vial, 0.4 mL containing biotinylated detection antibody in a protein-based buffer with a non-mercury preservative. Dilute prior to use in IGFBP-4 assay buffer. Store at 2-8°C until expiration date. Store at 2-8°C until expiration date.

SAR-126 Total IGFBP-4 Streptavidin-Enzyme Conjugate-Ready-to-Use (RTU)

One 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 2-8°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 buffered saline with a nonionic detergent. Store at 2-30°C until expiration date. Dilute 25-fold with deionized water prior to use.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Microplate absorbance 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. Optional related ELISA test kits for research purpose:
 - a) picoPAPP-A ELISA; AL-101
 - b) Intact IGFBP-4 ELISA; AL-128

WARNINGS AND PRECAUTIONS

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

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

WARNING: Potential Biohazardous Material

This reagent may contain some human source material (e.g. 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, 2007.¹⁶

WARNING: Potential Chemical Hazard

Some reagents in this kit contain Pro-Clean 400 and Sodium azide¹⁷ 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, which can be obtained at AnshLabs.com or by request.

SAMPLE COLLECTION

- a) Serum is the recommended sample type.
- b) 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.
- c) Samples must be stored at -20°C or -80°C to avoid loss of bioactivity and contamination.
- d) Avoid assaying lipemic, hemolyzed or icteric samples.
- e) Avoid repeated freezing and thawing of samples. Thaw samples no more than 3 times.
- f) 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.

PROCEDURAL NOTES

1. A thorough understanding of this package insert is necessary for successful use of the Total IGFBP-4 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. Care should be taken to add TMB into the wells to minimize potential assay drift due to variation in the TMB incubation time. Avoid exposure of the reagents to excessive heat or direct sunlight.

PREPARATION OF REAGENTS

- Total IGFBP-4 calibrators B-F and Total IGFBP-4 Controls I & II:** Tap and reconstitute IGFBP-4 Calibrator B-F and IGFBP-4 Controls I & II each with 0.5 mL deionized water. Solubilize, mix well and use after reconstitution.
- 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.
- 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.
- Total IGFBP-4 Antibody-Biotin Conjugate Solution:** The IGFBP-4 Antibody-Biotin Conjugate Concentrate should be diluted at a ratio of 1 part conjugate to 50 parts of IGFBP-4 Assay Buffer, according to the number of wells used. If an entire plate is to be used pipet exactly 220 μL of the Concentrate into 11 mL of the buffer.

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 prior to the assay.

- Reconstitute **Total IGFBP-4 Calibrator B-F and Total IGFBP-4 Controls I & II** each with **0.5 mL** deionized water. Solubilize for 10 minutes, Mix well by gentle vortex.
- Label the microtitration strips to be used.
- Pipette **25 μL** of the **Calibrator, Controls and samples** to the appropriate wells.
- Add **100 μL** of the **IGFBP-4 Assay Buffer** to each well using a repeater pipette.
- Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **60 minutes** at room temperature. ($23\pm 2^\circ\text{C}$)
- During the last **20-30 minutes** of the incubation, prepare the Total IGFBP-4 Antibody-Biotin Conjugate Solution by diluting the Total IGFBP-4 Biotin Conjugate Concentrate (BCC-126) in the Assay buffer as described under the Preparation of the Reagents section of this insert.
- Aspirate and wash each strip **5 times** with Wash Solution (350 μL /per well) using an automatic microplate washer.
- Add **100 μL** of the **IGFBP-4 Biotin Conjugate Solution** (As specified in Preparation of Regents, step 4) to each well using a repeater pipette.
- Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **60 minutes** at room temperature. ($23\pm 2^\circ\text{C}$)
- Aspirate and wash each strip **5 times** with the Wash Solution (350 μL /per well) using an automatic microplate washer.
- Add **100 μL** 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 μL /per well) using an automatic microplate washer.
- Add **100 μL** 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 **8-12 min** at room temperature.
NOTE: Visually monitor the color development to optimize the incubation time.
- 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

NOTE: The results in this package insert were calculated by plotting the **linear optical density (OD)** data on the **y-axis** and **log Total IGFBP-4 concentration** on **X-axis** using a non-linear regression curve-fit. Other data reduction methods may give slightly different results.

- Calculate the mean optical density (OD) for each calibrator, Control, or Unknown.
- Plot the mean OD readings for each of the Calibrators along the y-axis versus log of the Total IGFBP-4 concentrations in ng/mL along the x-axis, using a nonlinear curve-fit.
- Determine the Total IGFBP-4 concentrations of the Controls and unknowns from the calibration curve by matching their mean OD readings with the corresponding Total IGFBP-4 concentrations.
- Any sample reading higher than the highest Calibrator should be appropriately diluted with the 0 ng/mL (CAL A) and re-assayed.
- Any sample reading lower than the analytical sensitivity should be reported as such.

LIMITATIONS

The reagents supplied in this kit are optimized to measure Total IGFBP-4 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

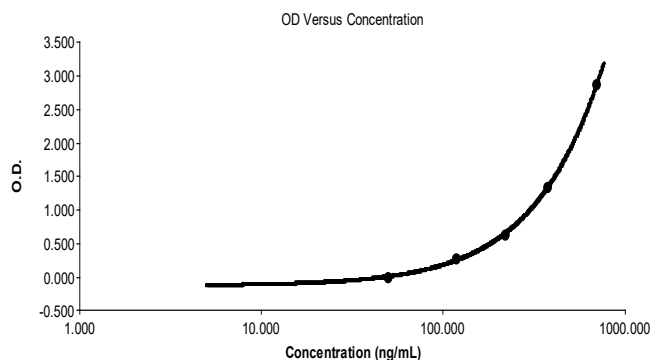
QUALITY CONTROL

- Each laboratory should establish mean values and acceptable ranges to assure proper performance.
- Total IGFBP-4 ELISA controls or other commercial controls should fall within established confidence limits.
- The confidence limits for Total IGFBP-4 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 Absorbance	Conc (ng/mL)
A1, A2	Calibrators A	0.016 (Blank)	0
B1, B2	B	0.079	50
C1, C2	C	0.288	117.9
D1, D2	D	0.673	218.7
E1, E2	E	1.453	375.8
F1, F2	F	2.842	702.2

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

Typical Calibration curve:**ANALYTICAL CHARACTERISTICS**

All analytical characteristics are stated in ng/mL.

Analytical Sensitivity:

The analytical sensitivity in the assay as calculated by the interpolation of mean plus two standard deviations of 12 runs with two replicates each (n=24) of calibrator A (0 ng/mL) and Calibrator B (50 ng/mL) is **4.735 ng/mL**.

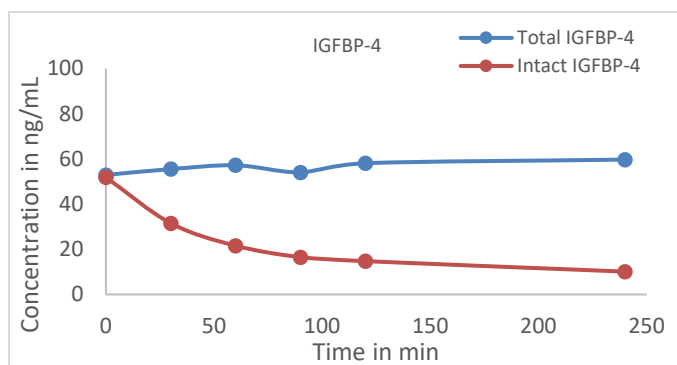
Cross reactivity and specificity:

The capture and detection antibody binds to the C terminal of the IGFBP-4 molecule. The monoclonal antibody pair used in the assay detects human, Equine, Canine, Bovine, Caprine and Rat IGFBP-4. Closely related analytes when tested in the assay in the concentrations shown in the table did not show any detectable cross reactivity.

Sample No.	Cross-reactant	Concentration (ng/mL)	% Cross-reactivity
1	IGF-I	1000	ND
2	IGFBP-2	1000	ND
3	IGFBP-3	1000	ND
4	IGFBP-3/IGF-1 complex	1000	ND
5	IGFBP-5	1000	ND
6	Rat IGF-I	1000	ND
7	IGF-II	1000	ND

Inhibition:

IGFBP-4 was incubated with active PAPP-A for up to six hours. Reaction mixture were collected and measured in Intact IGFBP-4 and Total IGFBP-4 ELISA assay at six different time points as shown below. No significant changes were observed in the Total IGFBP-4 concentration.

**Imprecision:**

Reproducibility of the Total IGFBP-4 assay was determined in a study using two controls and one serum pool. The study included a total of 12 assays, two replicates of each per assay (n=24). Representative data were calculated based on NCCLS EP5-A guidelines and are presented in the following table.

Sample	Mean conc. (ng/mL)	Within run		Between run		Total	
		SD	%CV	SD	%CV	SD	%CV
Control I	122.33	2.061	1.68%	4.182	3.42%	4.662	3.81%
Control II	363.28	12.584	3.46%	6.132	1.69%	13.999	3.85%
QC Pool	249.40	7.625	3.06%	10.105	4.05%	12.659	5.08%

Recovery:

Known amounts of IGFBP-4 antigen were added to three serum samples containing different levels of endogenous Total IGFBP-4. The concentration of Total IGFBP-4 was determined before and after the addition of exogenous IGFBP-4 antigen and the percent recovery was calculated.

Sample	Endogenous Conc. (ng/mL)	Expected Conc. (ng/mL)	Observed Conc. (ng/mL)	% Recovery
1	135.67	230.09	208.38	91%
		324.51	294.20	91%
		418.93	426.48	102%
2	161.64	251.73	213.32	85%
		341.83	320.32	94%
		431.92	422.74	98%
3	138.08	232.10	214.28	92%
		326.12	318.95	98%
		420.14	370.60	88%

Linearity:

Based on dilutions of the three serum samples containing various Total IGFBP-4 levels diluted with Calibrator A/sample diluent the % recovery on individual samples is represented in the following:

Sample	Dilution Factor	Expected Conc. (ng/mL)	Observed Conc. (ng/mL)	% Recovery
1	Neat	420.56	Neat	NA
	1:2	210.28	197.55	94%
	1:4	105.14	90.61	86%
2	Neat	507.31	Neat	NA
	1:2	253.65	265.42	105%
	1:4	126.83	146.73	116%
3	Neat	230.65	Neat	NA
	1:2	115.33	104.68	91%
	1:4	57.66	48.64	84%

Interference:

When potential interferents (hemoglobin, triglycerides and bilirubin) were added at least at two times their physiological concentration to control sample, Total IGFBP-4 concentration were within $\pm 10\%$ 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	132.13	138.85	5.09
		243.57	252.37	3.61
Triglycerides	5.00	132.13	128.46	-2.78
		243.57	244.49	0.38
Bilirubin	0.60	132.13	131.59	-0.41
		240.35	246.04	2.36

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

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