

Intact IGFBP-3 ELISA

RUO
AL-149

INTENDED USE

The Intact IGFBP-3 enzyme linked immunosorbent assay (ELISA) kit provides materials for the quantitative measurement of intact IGFBP-3 in human serum and other biological fluids. This kit is intended for laboratory Research Use Only and is not for use in diagnostic or therapeutic procedures.

SUMMARY

Insulin-like growth factor binding protein-3 (IGFBP-3) is a 264-amino acid peptide (MW 29 kD) produced by the liver as well as other tissues. It is the most abundant of a group of IGFBPs that transport, and control bioavailability and half-life of insulin-like growth factors (IGF), in particular IGF-I, the major mediator of the anabolic- and growth-promoting effects of growth hormone (GH).¹ The protein forms a ternary complex with insulin-like growth factor acid-labile subunit (IGFALS) and either insulin-like growth factor (IGF) I or II, thus prolonging the half-life of IGFs and altering their interaction with cell surface receptors. Most of the IGFBP-3 in plasma is present as the high molecular weight ternary complex, however, small amounts of free IGFBP-3 are also found.^{2,3}

IGFBP-3 also exhibits intrinsic growth-regulating effects that are not yet fully understood but have evoked interest with regards to a possible role of IGFBP-3 as a prognostic tumor marker. Low IGFBP-3 and IGF-I levels are observed in GH deficiency or GH resistance,⁴ also epidemiologic studies suggest that low IGFBP-3 is associated with greater risk of aggressive, metastatic prostate cancers.⁵ The elevated serum IGFBP-3 and IGF-I levels indicate a sustained overproduction of GH, or excessive rhGH therapy. Both conditions are associated with generalized organomegaly,⁶ hypertension,⁷ diabetes,⁸ cardiomyopathy,⁹ osteoarthritis,¹⁰ and diminished longevity. The Ansh Labs Intact IGFBP-3 Assay uses an acidification and neutralization method to dissociate Intact IGFBP-3 from all the binding subunits. Intact IGFBP-3 levels are quantified in the extracted samples using a highly sensitive and specific Intact IGFBP-3 ELISA. Total IGFBP-3 levels can be measured using Total IGFBP-3 ELISA (AL-120).

The concentration of bioactive IGF-I, total IGFBP-3 and intact IGFBP-3 in biological fluid can be measured accurately using immunoassay methods (Bioactive IGF-1 ELISA; AL-122, IGF-I inhibition mAb capture and IGF-1 C-terminal mAb) detection; Total IGFBP-3 ELISA; AL-120, C-terminal IGFBP-3 capture and detection mAb; and Intact IGFBP-3 ELISA; AL-149, C-terminal mAb capture and N-terminal mAb detection, respectively). The ratio of total to Intact IGFBP-3 concentration measured in individual subject over time will help normalize the IGFBP-3 variability between subjects. The immunoassay methods designed for the measurement of bioactive IGF-I, total and Intact IGFBP-3 in patient samples could be of practical value for the diagnosis or prediction of various pathologies including growth abnormalities and cancer.

PRINCIPLE OF THE TEST

The Intact IGFBP-3 ELISA is a quantitative two-step sandwich type immunoassay. The capture monoclonal antibody binds to the C terminal region whereas detection antibody binds to N terminal region of the IGFBP-3

molecule. In the first step Calibrators, Controls and unknown diluted samples are added to IGFBP-3 antibody coated micro titer wells and incubated. After first incubation and washing step, the wells are incubated with horseradish peroxidase labelled antibody conjugate. After a 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-HRP conjugate binds to the solid phase antibody-antigen complex. Finally, the antibody-antigen and conjugate complex bound to the well is detected by addition of 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 IGFBP-3 in the samples and calibrators.

MATERIALS SUPPLIED

CAL-149A

IGFBP-3 Calibrator A

One bottle, **25 mL**, labeled IGFBP-3 Cal A, containing 0 ng/mL IGFBP-3 in protein based buffer and Pro-Clean 400. Store unopened at 2-8°C until the expiration date.

CAL-149B - CAL-149F

Intact IGFBP-3 Calibrators B – F (Lyophilized)

Five vials, labeled B-F, containing concentrations of approximately 2.0 – 75.0 ng/mL recombinant IGFBP-3 derived from CHO cells, 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 use and discard after the run. Avoid repeated freeze thaws. The IGFBP-3 concentration in the IGFBP-3 calibrators is traceable to the manufacturer's working calibrators. Calibrators should not be diluted in IGFBP-3 Calibrators A. Calibrator concentrations are assigned based on serially diluted native human serum sample. Values assigned by other methodologies may be different. Such differences, if present, may be caused by inter-method bias.

CTR-149-I & CTR-149-II

Intact IGFBP-3 Controls I & II (Lyophilized)

Two vials, labeled Levels I and II containing low and high IGFBP-3 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 use and discard after the run. Avoid repeated freeze thaws.

PLT-120

IGFBP-3 Coated Microtitration strips

One strip holder, containing 12 strips and 96 microtitration wells with anti IGFBP-3 C-terminal 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.

SPB-149-I Intact IGFBP-3 Sample Buffer I

One bottle, 25 mL, containing sample buffer I with a non-mercury preservative. Store unopened at 2 to 8°C until the expiration date.

SPB-149-II Intact IGFBP-3 Sample Buffer II

One bottle, 25mL, containing sample buffer II with a non-mercury preservative. Store unopened at 2 to 8°C until the expiration date.

ASB-120 IGFBP-3 Assay Buffer

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

ECR-149 Intact IGFBP-3 Antibody-Enzyme Conjugate Ready-to-Use

One bottle, 12 mL, containing anti IGFBP-3 N-terminal antibody conjugated to HRP in a protein buffer with a non-mercury preservative. Store at 2 to 8°C until the 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. Microtitration plate 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 μ L.
5. Repeater pipette.
6. Culture tubes for sample pretreatment.
7. Tight-fitting tube rack
8. Vortex mixer.
9. Deionized water.

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, 2007.¹¹

WARNING: Potential Chemical Hazard

Some reagents in this kit contain Pro-Clean 400 and Sodium azide¹² 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) 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 may be stored at 4°C if assayed within 24 hours; otherwise samples must be stored at -20°C or below 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 intact IGFBP-3 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. 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

1. **IGFBP-3 calibrators B-F and IGFBP-3 Controls I & II:** Tap and reconstitute IGFBP-3 Calibrator B-F and IGFBP-3 Controls I & II each with 0.5 mL deionized water. Solubilize the calibrators and controls for 15 mins in deionized water and mix well before use.
2. **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.
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.

SAMPLE PREPARATION

SAMPLE PRE-TREATMENT AND STABILIZATION:

Note: Calibrators and Controls upon reconstitution are ready to use and should not be diluted 1:40.

- For each unknown sample, label one 12x75mm culture tube appropriately and add **95 µL of Intact IGFBP-3 Sample Buffer I**.
- Pipette **10 µL of sample** into the appropriate pre-labeled tubes.
- Place the tubes in a tight-fitting tube rack and shake at a slow speed (300-400 rpm) at room temperature ($23 \pm 2^\circ\text{C}$) for **30 minutes**.
- Pipette **95 µL of Intact IGFBP-3 Sample Buffer II** into each tube.
- Add **200 µL of CAL-149A** into each tube and shake at a slow speed (300-400 rpm) at room temperature ($23 \pm 2^\circ\text{C}$) for **10 minutes**.
- Vortex well. The samples are now ready to be assayed.

Note: Any samples reading higher than the highest calibrator should be pre-treated first (steps a-f) and diluted in calibrator A.

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: Any samples reading higher than the highest calibrator should be pre-treated first and diluted in 0 ng/mL (CAL A) and re-assayed.

- Label the microtitration strips to be used.
- Pipette **25 µL of the Calibrator and Controls and treated Unknowns (see sample preparation section)** to the appropriate wells.
- Add **100 µL** of the IGFBP-3 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 ($23 \pm 2^\circ\text{C}$).
- Aspirate and wash each strip **5 times (350 µL/per well)** with Wash Solution using an automatic microplate washer.
- Add **100 µL** of the IGFBP-3 Enzyme Conjugate Solution (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 precision 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 ($23 \pm 2^\circ\text{C}$).

NOTE: Visually monitor the color development to optimize the incubation time.

- 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: 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 log optical density (OD) data on the y-axis and log IGFBP-3 concentration on X-axis using a linear regression curve-fit.

- Optimum results can be obtained at incubation temperature of (**$23 \pm 2^\circ\text{C}$**).
- Calculate the mean optical density (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 Intact IGFBP-3 concentrations in ng/mL along the x-axis, using a Linear regression curve-fit.
- Determine the Intact IGFBP-3 concentrations of the Controls and unknowns from the calibration curve by matching their mean OD readings with the corresponding Intact IGFBP-3 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.
- Multiply the measured concentrations in ng/mL by the dilution factor (40X).**

LIMITATIONS

The reagents supplied in this kit are optimized to measure intact IGFBP-3 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.¹³

QUALITY CONTROL

- Each laboratory should establish mean values and acceptable ranges to assure proper performance.
- Intact IGFBP-3 ELISA controls or other commercial controls should fall within established confidence limits.
- The confidence limits for IGFBP-3 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 (ng/mL)
A1, A2	A	0.017 (Blank)	0
B1, B2	B	0.050	2.2
C1, C2	C	0.107	4.2
D1, D2	D	0.22	7.7
E1, E2	E	0.843	27.0
F1, F2	F	4.013	87.0

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

ANALYTICAL CHARACTERISTICS

All analytical characteristics are stated ng/mL.

Limit of Detection (LoD): The analytical sensitivity in the assay as calculated by the interpolation of mean plus two standard deviation of 32 replicates in 8 runs of calibrator A (0 ng/mL) and calibrator B (5 ng/mL) is **1.37 ng/mL**.

Imprecision: Reproducibility of the intact IGFBP-3 assay was determined in a study using serum pools. The study included a total of 8 assays, four replicates of each per assay (n=32). Representative data are presented in the following table.

Sample	Mean conc.	Within run		Between run		Total	
	(ng/mL)	SD	%CV	SD	%CV	SD	%CV
Sample 1	22.07	1.09	4.93	0.751	3.40	1.322	5.99
Sample 2	54.47	1.57	2.88	1.660	3.05	2.283	4.19
Sample 3	64.26	1.05	1.64	2.728	4.25	2.924	4.55
Sample 4	74.38	1.40	1.88	3.752	5.04	4.003	5.38

Analytical Specificity:

The monoclonal antibody pair used in the assay detects Intact-IGFBP-3. Other related analytes at 1000 ng/mL concentration did not show any cross-reaction.

Cross-reactant	Concentration (ng/mL)	% Cross-reactivity
IGF-I	1000	ND
IGFBP-2	1000	ND
N-term IGFBP-3 C-term IGFBP-3	2000	100%
IGFBP-4	1000	ND
IGFBP-5	1000	ND
Rat IGF-I	1000	ND
IGF-II	1000	ND
Pregnancy Sera (16 WK GST)	NA	ND

Linearity:

Four serum samples were diluted at 1:80, 1:160 and 1:320 as per the procedure below. For a 1:40 dilution, 10 µL of sample was added to 95 µL sample buffer I + 95 µL sample buffer II and 200 µL of CAL-149A as per the sample preparation protocol. Additional Dilution levels were done by diluting the pretreated sample (following steps 1-5 in sample preparation section) with calibrator A. The % recovery is shown in table below.

Sample	Dilution Factor	Expected Conc. (ng/mL)	Observed Conc. (ng/mL)	% Recovery
1	1:40	21.74	NA	NA
	1:80	10.87	10.79	99%
	1:160	5.43	4.69	86%
	1:320	2.72	2.22	82%
2	1:40	18.15	NA	NA
	1:80	9.08	9.58	105%
	1:160	4.54	4.26	94%
	1:320	2.27	1.87	82%
3	1:40	12.87	NA	NA
	1:80	6.44	6.49	101%
	1:160	3.22	2.81	87%
	1:320	1.61	1.36	84%

Interference:

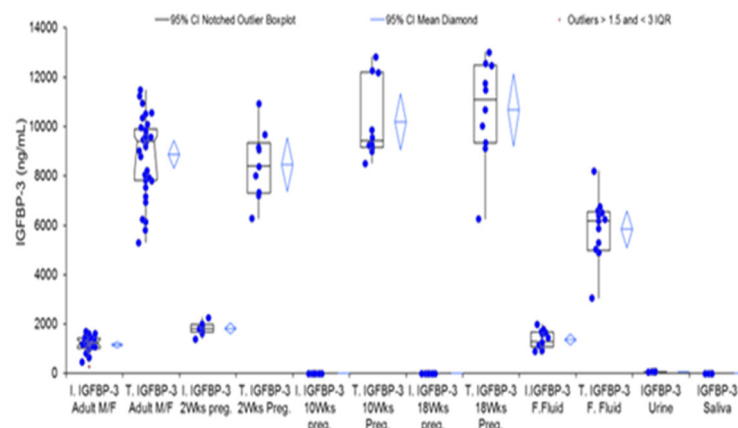
When potential interferents were tested at the concentration listed in the table below the % difference in sample concentration with respect to control sample were within 11%.

Interferents	Sample #	Sample (ng/mL)	Dosed Sample (ng/mL)	% Recovery	% Difference
9.3 mg/mL Hemoglobin	1	78.29	86.55	110.56	10.56
	2	176.52	172.21	97.56	-2.44
	3	65.06	64.73	99.49	-0.51
	4	94.21	88.98	94.46	-5.54
51 mg/mL Triglycerides	1	78.29	84.84	108.37	8.37
	2	176.52	178.16	100.93	0.93
	3	65.06	65.58	100.80	0.80
	4	95.21	95.93	101.83	1.83
4.2 mg/mL Bilirubin	1	87.04	85.03	97.69	-2.31
	2	183.22	181.05	98.82	-1.18
	3	66.50	65.45	98.43	-1.57
	4	98.29	96.29	97.96	-2.04

Reference Ranges:

Intact and Total IGFBP-3 in biological fluids are shown in the figure below:

IGFBP-3 measured in T = Total IGFBP-3, I= Intact IGFBP-3, M/F= adult Male/Females, Preg= Gestational Weeks, F. Fluid = Follicular Fluids.

**Expected Values:**

The expected ranges for Intact IGFBP-3 in pediatric male samples in the age range of 3.0 – 18.0 years were calculated using 95% non-parametric estimation. A total of 404 samples in Pubic Hair Tanner stages 1 - 5 were evaluated using Analyse-It® for Microsoft Excel as seen in table below.

Pubic Hair Tanner Stage	No of specimens (n)	Median Conc. (ng/mL)	Intact IGFBP-3 (ng/mL) 95% CI
1	218	1151.0	367.0 - 1970.0
2	54	1614.0	948.0 - 2168.0
3	32	1873.0	1081.0 - 2279.0
4	50	1895.0	1251.0 - 2714.0
5	50	2006.0	1063.0 - 2892.0

The expected ranges for Intact IGFBP-3 in pediatric female samples in the age range of 2.4 – 18.0 years were calculated using 95% non-parametric estimation. A total of 432 samples in Breast Tanner stages 0 - 5 were evaluated using Analyse-It® for Microsoft Excel as seen in table below.

Breast Tanner Stage	No of specimens (n)	Median Conc. (ng/mL)	Intact IGFBP-3 (ng/mL) 95% CI
0	15	955.0	504.0 - 2017.0
1	174	1108.0	351.0 - 1775.0
2	61	1478.0	646.0 - 2013.0
3	58	1709.0	936.0 - 2620.0
4	53	1916.0	1141.0 - 2525.0
5	71	1887.0	1062.0 - 2797.0

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.

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