Total IGFBP-3 ELISA

RUO

AL-120

INTENDED USE

The Total IGFBP-3 enzyme linked immunosorbent assay (ELISA) kit provides materials for the quantitative measurement of Total 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. 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-1, the major mediator of the anabolic- and growth-promoting effects of growth hormone (GH). 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.[1,2]

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-1 levels are observed in GH deficiency or GH resistance,[3] also epidemiologic studies suggest that low IGFBP-3 is associated with greater risk of aggressive, metastatic prostate cancers.[4] The elevated serum IGFBP-3 and IGF-1 levels indicate a sustained overproduction of GH, or excessive rhGH therapy. Both conditions are associated with generalized organomegaly,[5] hypertension,[6] diabetes,[7] cardiomyopathy,[8] osteoarthritis, [9] and diminished longevity.

The Ansh Labs Total IGFBP-3 Assay uses an acidification and neutralization method to dissociate IGFBP-3 from all the binding subunits. Total IGFBP-3 levels are quantified in the extracted samples using a highly sensitive and specific total IGFBP-3 ELISA. Intact IGFBP-3 levels can be measured using Intact IGFBP-3 ELISA (AL-149).

PRINCIPLE OF THE TEST

The Total IGFBP-3 ELISA is a quantitative two-step sandwich type immunoassay. The capture and detection antibodies bind to the C terminal of the IGFBP-3 molecule. In the first step Calibrators, Controls and unknown treated samples are added to C-terminal IGFBP-3 antibody coated micro titer wells and incubated. After the first incubation and washing step, the wells are incubated with horseradish peroxidase labelled C-terminal IGFBP-3 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 Total IGFBP-3 in the samples and calibrators.

MATERIALS SUPPLIED

CAL-120A IGFBP-3 Calibrator A

One bottle, 2.5 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-120B - Cal-120F IGFBP-3 Calibrators B – F (Lyophilized)

Five vials, labeled B-F, containing concentrations of approximately 10-250 ng/mL IGFBP-3 in 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-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. Individual calibrator concentrations are assigned based on native total IGFBP-3 linearity of dilution. Values assigned by other methodologies may be different. Such differences, if present, may be caused by inter-method bias.

CTR-120-I & CTR-120-II 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.

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.

SPB-121-I-42mL IGF-I Sample Buffer I-42 mL

One bottle, 42 mL, containing sample buffer I with a non-mercury preservative. Store unopened at 2 to 8°C until the expiration date.

SPB-120-II-42mL IGFBP-3 Sample Buffer II-42 mL

One bottle, 42mL, containing sample buffer II with a non-mercury preservative. Store unopened at 2 to 8°C until the expiration date.

ECR-120 Total IGFBP-3 Antibody-Enzyme Conjugate Ready-to-Use

One bottle, 12 mL, containing IGFBP-3 C-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 µL.
5. Repeater pipette.
6. Vortex mixer.
7. Disposable 12 x 75 mm culture tubes
8. Deionized water.
9. Intact IGFBP-3 levels can be measured by AL-149 ELISA.

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.[10]

WARNING: Potential Chemical Hazard

Some reagents in this kit contain Pro-Clean 400 and Sodium azide[11] 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 Total IGFBP-3 ELISA assay. 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 components 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 minutes 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 ± 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.

SAMPLE PREPARATION

SAMPLE PREPARATION (1:100 dilution):

Note: Calibrators and Controls upon reconstitution are ready to use and should not be diluted 1:100.

a) For each unknown sample, label one 12x75mm culture tube appropriately and add **495 µL of IGF-I Sample Buffer I.**

b) Pipette **10 µL of each sample** into the appropriate pre-labeled tubes.
c) Place the tubes in a tight-fitting tube rack and shake at a slow speed (300-400 rpm) at room temperature (23 ± 2°C) for **30 minutes**.
d) Pipette **495 µL** of **IGFBP-3 Sample Buffer II** into each tube and shake at a slow speed (300-400 rpm) at room temperature (23 ± 2°C) for **10 minutes**.
e) Vortex well. The samples are now ready to be assayed.

Note: Any sample reading higher than the highest calibrator or reading lower than the lowest calibrator should be further diluted or spiked as per the procedure listed in following table.

Spike/Dilution	Sample Buffer I Volume	Sample Volume	Sample Buffer II Volume
1:50 (Spiking)	980.0 µL	40.0 µL	980.0 µL
1:100 (Baseline)	990.0 µL	20.0 µL	990.0 µL
1:200 (Dilution)	995.0 µL	10.0 µL	995.0 µL

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.

1. Prepare all samples to be assayed as per the "Sample Preparation" section of this package inserts.
2. Label the microtitration strips to be used.
3. Pipette **25 µL** of the **Calibrator, Controls, and treated Unknowns** to the appropriate wells.
4. Add **100 µL** of the **IGFBP-3 Assay Buffer** 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 6**0 minutes** at room temperature (23 ± 2°C).
6. Aspirate and wash each strip **5 times** with Wash Solution **(350 µL /per well)** using an automatic microplate washer.
7. Add **100 µL** of the **Total IGFBP-3 Enzyme Conjugate Solution (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 **8-12 min** at room temperature (23 ± 2°C).
    NOTE: *Visually monitor the color development to optimize the incubation time.*
12. Add **100 µL** of the S**topping 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 **log optical density (OD) data on the y-axis and log IGFBP-3 concentration on X-axis** using a cubic regression curve-fit. Alternatively, log vs. log linear regression curve-fit can be used. Other data reduction methods may give slightly different results.*

1. Optimum results can be obtained at incubation temperature of **(23 ± 2°C)**.
2. Calculate the mean optical density (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 Total IGFBP-3 concentrations in ng/mL along the x-axis, using a cubic regression curve-fit.
4. Determine the Total IGFBP-3 concentrations of the Controls and unknowns from the calibration curve by matching their mean OD readings with the corresponding Total IGFBP-3 concentrations.
5. Any sample reading higher than the highest Calibrator should be appropriately diluted with the 0 ng/mL (Calibrator A) and re-assayed.
6. Any sample reading lower than the analytical sensitivity should be reported as such.
7. **Multiply the measured concentrations in ng/mL by the dilution factor (100X)**.

LIMITATIONS

The reagents supplied in this kit are optimized to measure Total 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.[12]

QUALITY CONTROL

- Each laboratory should establish mean values and acceptable ranges to assure proper performance.
- Total IGFBP-3 ELISA controls or other commercial controls should fall within established confidence limits.
- The confidence limits for Total 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 Absorbance	Conc. (ng/mL)
A1, A2	A	0.002 (Blank)	0
B1, B2	B	0.049	2.0
C1, C2	C	0.271	11.4
D1, D2	D	1.001	49.0
E1, E2	E	2.222	136.0
F1, F2	F	3.369	240.0

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.

Analytical Sensitivity:

The analytical sensitivity in the assay as calculated by the interpolation of mean plus two standard deviation of 16 replicates of calibrator A (0 ng/mL) and calibrator B (2 ng/mL) is **0.19 ng/mL.**

Imprecision:

Reproducibility of the Total IGFBP-3 ELISA assay was determined in a study using four QC serum pools. The study included a total of 16 assays, two replicates of each per assay (n=32). 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
1	93.4	5.4	5.8%	3.7	3.9%	6.5	7.0%
2	70.6	4.1	5.9%	4.1	5.8%	5.8	8.2%
3	98.4	5.6	5.7%	4.1	4.2%	6.9	7.0%
4	51.3	3.3	6.4%	4.6	9.1%	5.7	11.1%

Analytical Specificity:

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

Sample No.	Cross-reactant	Concentration (ng/mL)	% Cross-reactivity
1	Rat IGF-I	1000	ND
2	IGF-I	1000	ND
3	IGFBP-2	1000	ND
4	IGFBP-3/IGF-I complex	1000	20
5	IGFBP-4	1000	ND
6	IGFBP-5	1000	ND
7	IGF-II	1000	ND

ND: Not-detected

The antibody pair used in Total IGFBP-3 assay detects monkey samples. The assay does not detect Bovine, Canine, Equine, Ovine, Rabbit, Porcine, Goat, and Mouse species.

Interference:

When hemoglobin, biotin, intralipids and bilirubin were added at a concentration greater than two folds of their physiological concentration to control sample, average Total IGFBP-3 concentration were within ± 15% of the control as represented in the following table.

Interferent	Interferent Dose	Analyte Conc. (ng/mL)	Spiked Sample Conc. (ng/mL)	% Difference
Hemoglobin	1 mg/mL	93.89	95.38	1.6
	0.5 mg/mL	98.43	93.99	-4.5
	0.1 mg/mL	96.78	101.10	4.5
Hemoglobin	1 mg/mL	58.76	59.28	0.9
	0.5 mg/mL	62.06	66.32	6.9
	0.1 mg/mL	65.96	65.76	-0.3
Biotin	1200 ng/mL	86.64	91.09	5.1
	600 ng/mL	94.49	85.78	-9.2
	200 ng/mL	102.68	107.40	4.6
Biotin	1200 ng/mL	67.50	63.83	-5.4
	600 ng/mL	65.30	64.77	-0.8
	200 ng/mL	67.17	69.86	4.0
Intralipids	20 mg/mL	109.78	114.21	4.0
	10 mg/mL	120.76	120.32	-0.4
	5 mg/mL	113.03	117.74	4.2
Intralipids	20 mg/mL	65.83	72.38	10.0
	10 mg/mL	74.28	73.14	-1.5
	5 mg/mL	75.41	73.59	-2.4
Bilirubin	0.66 mg/mL	89.13	82.50	-7.4
	0.2 mg/mL	42.59	42.03	-1.3
Bilirubin	0.66 mg/mL	117.04	111.24	-5.0
	0.2 mg/mL	68.43	68.05	-0.6

Spike Recovery and Linearity of Dilution:

Six serum samples were treated, and each level of sample spike/dilution was independently prepared as mentioned in the sample preparation section and assayed for spike recovery and linearity of dilution as shown in table below.

Spike/Dilution	Sample Volume	Sample Buffer I Volume	Sample Buffer II Volume
1:50 (Spiking)	980.0 µL	40.0 µL	980.0 µL
1:100 (Baseline)	990.0 µL	20.0 µL	990.0 µL
1:200 (Dilution)	995.0 µL	10.0 µL	995.0 µL

For a spike recovery (1:50): 40 µL of sample was treated with 980 µL of IGF-1 Sample Buffer I and 980 µL of IGFBP-3 Sample Buffer II and assayed as per the procedure.

For sample dilution (1:200): 10 µL of sample was treated with 995 µL of IGF-1 Sample Buffer I and 995 µL of IGFBP-3 Sample Buffer II and assayed as per the procedure.

The expected concentrations for spiking recovery (1:50) and dilution recovery (1:200) were calculated from the baseline sample dilution (1:100) concentration. The % recovery is represented in the table below.

Sample	Dilution factor (1 in X)	Expected Value (ng/mL)	Observed Value (ng/mL)	% Recovery
1	1:50 (Spiking)	100.550	96.121	96%
	1:100 (Baseline)	50.275	n/a	n/a
	1:200 (Dilution)	25.138	27.253	108%
2	1:50 (Spiking)	134.606	133.358	99%
	1:100 (Baseline)	67.303	n/a	n/a
	1:200 (Dilution)	33.652	37.057	110%
3	1:50 (Spiking)	120.504	113.487	94%
	1:100 (Baseline)	60.252	n/a	n/a
	1:200 (Dilution)	30.126	30.765	102%
4	1:50 (Spiking)	84.728	74.891	88%
	1:100 (Baseline)	42.364	n/a	n/a
	1:200 (Dilution)	21.182	22.075	104%
5	1:50 (Spiking)	120.566	109.912	91%
	1:100 (Baseline)	60.283	n/a	n/a
	1:200 (Dilution)	30.142	30.6	102%
6	1:50 (Spiking)	82.420	81.126	98%
	1:100 (Baseline)	41.21	n/a	n/a
	1:200 (Dilution)	20.605	22.638	110%

Note: Since antigen is not treated with sample buffers, calibrator F should not be diluted by itself or used to spike samples.

Expected Values:

The expected ranges for Total 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)	Total IGFBP-3 (ng/mL) 95% CI
1	218	5118.0	2267.0 - 7853.0
2	54	5859.0	2779.0 - 8247.0
3	32	5265.0	3221.0 - 8703.0
4	50	4890.0	2361.0 - 9357.0
5	50	4262.0	2361.0 - 7718.0

The expected ranges for Total 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)	Total IGFBP-3 (ng/mL) 95% CI
0	15	5670.0	3986.0 - 7340.0
1	174	6008.0	2797.0 - 10106.0
2	61	6629.0	3268.0 - 10881.0
3	58	7001.0	4050.0 - 11132.0
4	53	5390.0	3227.0 - 10792.0
5	71	5161.0	2267.0 - 8392.0

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