Total Rat/Mouse IGFBP-5 ELISA RUO

AL-1026

INTENDED USE

The Total Rat/Mouse IGFBP-5 enzyme linked immunosorbent assay (ELISA) kit provides materials for the quantitative measurement of IGFBP-5 in mouse and rat samples. 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 5 (IGFBP-5) is part of the IGF-binding proteins that regulates IGF activity by binding to IGF ligands. The IGF axis consists of six high-affinity IGF-binding proteins (IGFBP 1-6). IGFBP-5 has 272 amino acids and weighs about 31 kDa. IGF axis is the principal endocrine system that regulates the differentiation, development, and maintenance of bone tissue. Dysfunction of this axis is associated with various skeletal pathologies such as growth disorders and abnormal bone structure.[1,2]

Pregnancy-Associated Plasma Protein-A2 (PAPP-A2) plays a major role in bone cell physiology as well. PAPP-A2 is also known to proteolyze IGFBP-5. Certain mutation in the PAPP-A2 protein inhibits its ability to proteolyze IGFBP-5. Consequently, affected individuals show microcephaly, mild BMD effects and thin long bones. Thus, IGFBP-5 plays an important role in osteoblast differentiation and bone tissue metabolism which remains to be further expounded upon.[1,2]

PRINCIPLE OF THE TEST

The Total Rat/Mouse IGFBP-5 ELISA is a quantitative two-step sandwich type immunoassay. In the first step, Calibrators, Controls and unknown diluted samples are added to IGFBP-5 antibody coated microtiter wells and incubated. After the 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-5 in the samples and calibrators.

MATERIALS SUPPLIED

CAL-1026A IGFBP-5 Calibrator A/Sample Diluent

One vial, 10 mL, labeled IGFBP-5 Cal A/Sample Diluent, containing 0 ng/mL IGFBP-5 in protein-based buffer and ProClin 300. Store unopened at 2-8°C until the expiration date.

CAL-1026B – CAL-1026F IGFBP-5 Calibrators B – F (Lyophilized)

Five vials, labeled B-F, containing concentrations of approximately 9.8-250 ng/mL IGFBP-5 in protein-based buffer and ProClin 300. 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 eppendorf vials immediately for multiple use and discard after the run. Calibrators are stable for up to four freeze thaws. The IGFBP-5 concentration in the IGFBP-5 calibrators is traceable to R&D System mouse IGFBP-5 antigen. Values assigned by other methodologies may be different. Such differences, if present, may be caused by inter-method bias.

CTR-1026-I & CTR-1026-II IGFBP-5 Controls I & II

Two vials, labeled Levels I and II containing low and high IGFBP-5 concentrations in protein-based buffer and ProClin 300. 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 eppendorf vials for multiple use. Stable for up to four freeze thaws.

PLT-1026 Total Rat/Mouse IGFBP-5 Coated Microtitration strips

One stripholder, containing 12 strips and 96 microtitration wells with IGFBP-5 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-1026 Total Rat/Mouse IGFBP-5 Assay Buffer

One bottle, 12 mL with a non-mercury preservative. Store at 2-8°C until expiration date.

CND-1026 Total Rat/Mouse IGFBP-5 Conjugate Diluent

One amber bottle, 12 mL, HRP in a buffer with a non-mercury preservative. Store at 2 to 8°C until the expiration date.

ECC-1026 Total Rat/Mouse IGFBP-5 Enzyme Conjugate Concentrate (50X)

One vial, 0.4 mL, containing IGFBP-5 antibody conjugated to HRP in a protein-based buffer with a non-mercury preservative. Store at 2 to 8°C until the expiration date. Dilute 10 – 30 minutes prior to use in Total Rat/Mouse conjugate diluent.

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, 405nm and 630 nm.
2. Microplate orbital shaker.
3. Microplate washer.
4. Semi-automated/manual precision pipette to deliver 10–250 µL.
5. Repeator pipette.
6. Vortex mixer.
7. 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," 6th Edition, 2020[3].

WARNING: Potential Chemical Hazard

Some reagents in this kit contain ProClin 300 and Sodium Azide[4] as a preservative. ProClin 300 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

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 -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 IGFBP-5 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. 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-5 calibrators B-F and IGFBP-5 Controls I & II**: Tap and reconstitute IGFBP-5 Calibrator B-F and IGFBP-5 Controls I & II each **with 0.5 mL deionized water**. Solubilize, 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 a resealable pouch with a desiccant. The pouch must be resealed to protect it from moisture.
4. **Total Rat/Mouse Enzyme Conjugate Solution:** The Total Rat/Mouse IGFBP-5 Enzyme Conjugate Concentrate should be diluted at a ratio of 1 part conjugate to 50 parts of Total Rat/Mouse IGFBP-5 Conjugate Diluent, according to the number of wells used. If entire plate is to be used pipet exactly 220µL of the concentrate into 11 mL of the Diluent.

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. Specimens reading higher than the last calibrator should be diluted with sample diluent.

Note: Do not dilute the calibrators and controls. All Rat samples should be diluted 1:25 and all mouse samples should be diluted 1:10 prior to assay.

1. Label the microtitration strips to be used.
2. **Sample Preparation:**
   a) For Rat Samples: 1:25 dilution
      i) For each unknown rat sample, label one eppendorf vial appropriately and add 75 µL of Calibrator A/Sample Diluent to each vial.
      ii) Add 3 µL of the rat specimens to the pre-labeled vials and vortex well.
   a) For Mouse Samples: 1:10 dilution
      i) For each unknown mouse sample, label one eppendorf vial appropriately and add 45 µL of Calibrator A/Sample Diluent to each vial.
      ii) Add 5 µL of the mouse specimens to the pre-labeled vials and vortex well.
3. Pipette **20 µL** of the **Calibrator, Controls and Unknowns** to the appropriate wells
4. Add **100 µL** of the Total Rat/Mouse IGFBP-5 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 **60 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 Rat/Mouse IGFBP-5 Enzyme Conjugate to each well using a repeater pipette. (Prepare the Total Rat/Mouse IGFBP-5

Enzyme Conjugate solution by diluting the total Rat/Mouse IGFBP-5 Enzyme Conjugate Concentrate in Total Rat/Mouse IGFBP-5 Conjugate Diluent as described under the preparation of the reagent section of this package insert.)

8. Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **60 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 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 an 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-5 concentration on X-axis** using a cubic regression curve-fit. Alternatively, log vs. log quadratic regression curve-fit can be used. Other data reduction methods may give slightly different results.*

1. Calculate the mean optical density (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 IGFBP-5 concentrations in ng/mL along the x-axis, using a cubic regression curve-fit.
3. Determine the IGFBP-5 concentrations of the Controls and unknowns from the calibration curve by matching their mean OD readings with the corresponding IGFBP-5 concentrations.
4. Any sample reading higher than the highest Calibrator should be appropriately diluted with the 0 ng/mL (CAL A / Sample Diluent) and re-assayed.
5. Any sample reading lower than the analytical sensitivity should be reported as such.
6. Multiply the measured concentration in ng/mL by the **dilution factor specified in step 2.**

LIMITATIONS

The reagents supplied in this kit are optimized to measure rat/moue IGFBP-5 levels in 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.[5]

QUALITY CONTROL

- Each laboratory should establish mean values and acceptable ranges to assure proper performance.
- Total Rat/Mouse IGFBP-5 ELISA controls or other commercial controls should fall within established confidence limits.
- The confidence limits for IGFBP-5 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	0.028 (Blank)	0
B1, B2	B	0.120	9.8
C1, C2	C	0.325	23.0
D1, D2	D	0.845	50.0
E1, E2	E	2.125	110.0
F1, F2	F	3.712	250.0

***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 in ng/mL.

Analytical Specificity:

The Analytical sensitivity or Minimum Detectable Dose (MDD) for Total Rat/Mouse IGFBP-5 ELISA was calculated from six assay runs with eight replicates (n=48) of calibrator A and two replicates (n=12) as per EP10A-3 and was determined to be 0.40 ng/mL.

Imprecision:

Reproducibility of the Total Rat/Mouse IGFBP-5 assay was determined in a study using samples in the low, mid, and high range. The study included a total of 5 assays, 3 replicates of each per assay (n=15). Representative data were calculated based on EP10A-3 guidelines and are presented in the following table.

Sample	Mean Conc. (ng/mL)	SD	%CV
1	15.0	0.30	2.03%
2	45.5	1.24	2.73%
3	116.6	5.82	4.99%

Cross reactivity and specificity:

The monoclonal antibody pair used in the assay detects IGFBP-5. 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	mouse IGFBP-2	50 ng/mL	Non-Detectable
2	mouse IGFBP-3	50 ng/mL	Non-Detectable
3	mouse IGFBP-4	50 ng/mL	Non-Detectable
4	mouse IGFBP-5 (R&D System)	50 ng/mL	200.0%
5	mouse IGF-I	50 ng/mL	Non-Detectable
6	mouse IGF-II	50 ng/mL	Non-Detectable
7	Rat IGF-II	50 ng/mL	Non-Detectable
8	Rat IGF-I	50 ng/mL	Non-Detectable

Species Immunoreactivity:

The antibody pair used in Total Rat/Mouse IGFBP-5 ELISA assay detects Rabbit, Goat, Bovine, Equine, Feline, Sheep, Ovine, Porcine, Mouse, Rat, Squirrel Monkey and Vervet Monkey samples.

Sample#	Species	Type	Dilution	O.D.	Observed Conc. (ng/mL)	Conc. (ng/mL)
1	Rabbit	Serum	10	1.21	70.4	703.7
2	Rabbit	Serum	10	1.83	104.0	1040.1
3	Rabbit	Serum	10	1.77	100.8	1007.8
4	Goat	Serum	10	0.59	38.3	383.0
5	Goat	Serum	10	0.77	48.1	480.6
6	Goat	Serum	10	0.22	16.7	166.6
7	Bovine	Serum	10	0.93	55.9	559.3
8	Bovine	Serum	10	0.84	51.4	514.4
9	Bovine	Serum	10	1.06	63.1	630.8
10	Canine	Tissue Extract	10	0.04	<7.089	ND
11	Canine	Tissue Extract	10	0.08	<7.089	ND
12	Canine	Serum	10	0.75	46.6	466.0

13	Canine	Serum	10	2.13	122.2	1221.5
14	Equine	Cyst Fluid	50	0.36	25.5	1276.2
15	Equine	Serum	10	1.57	89.9	899.3
16	Equine	Serum	10	0.45	30.5	305.2
17	Equine	Serum	10	1.20	70.1	700.6
18	Feline	Serum	10	1.17	68.7	687.2
19	Feline	Serum	10	0.29	21.1	211.0
20	Sheep	Serum	10	1.66	94.4	943.6
21	Ovine	Serum	10	1.29	74.9	748.6
22	Ovine	Serum	10	0.77	48.0	479.6
23	Porcine	Serum	10	1.29	74.7	747.0
24	Porcine	Serum	10	1.37	79.0	789.6
25	Mouse	Serum	10	2.26	130.4	1303.9
26	Mouse	Serum	10	2.01	114.6	1146.3
27	Mouse	Serum	10	1.59	90.7	906.9
28	Rat	Serum	25	0.35	24.9	621.6
29	Rat	Serum	25	1.25	72.7	1817.9
30	Rat	Serum	25	0.74	46.4	1159.9
31	Squirrel Monkey	Serum	10	1.28	74.1	740.7
32	Vervet Monkey	Serum	10	2.73	162.5	1625.3

ND= Not detectable

Linearity:

Calibrator F and two rat samples containing various IGFBP-5 levels were serially diluted in calibrator A/sample diluent. The % recovery on individual samples is represented in the following table.

Sample ID	Dilution factor (1 in X)	Expected Value in ng/mL	Observed Value in ng/mL	%Recovery
Cal. F	Neat	250.00		
	2	125.00	132.0	106%
	4	62.50	62.5	100%
	8	31.25	30.1	96%
	16	15.63	15.7	100%
	32	7.81	8.1	104%
1	Neat, 1:25	83.92		
	50	41.96	44.2	105%
	100	20.98	23.9	114%
2	Neat, 1:25	41.91		
	50	20.95	23.9	114%
	100	10.48	12.2	116%

Spike Recovery

Known amounts of IGFBP-5 antigen were added to four serum samples containing different levels of endogenous IGFBP-5. The concentration of IGFBP-5 was determined before and after the addition of exogenous IGFBP-5 and the percent recovery was calculated.

Sample ID	Endogenous Value in ng/mL	Expected in ng/mL	Observed in ng/mL	Average %Recovery
S1	28.078	32.2	31.7	**96%**
		36.3	34.3	
		40.4	38.2	
S2	25.38	29.6	28.6	**97%**
		33.9	33.2	
		38.2	37.2	
S3	46.26	49.5	49.5	**99%**
		52.7	51.2	
		55.9	55.1	
S4	32.25	36.2	34.3	**92%**
		40.1	35.8	
		44.0	40.0	

Interference:

When potential interferents (hemoglobin, biotin, intralipids, and bilirubin) were added at least at two times their physiological concentration to control sample, average IGFBP-5 concentrations were within ± 15% of the control as represented in the following table.

Interferent	Interferent Dose	Sample IGFBP-5 (ng/mL)	Dosed Sample IGFBP-5 (ng/mL)	% Difference
Hemoglobin	1 mg/mL	504.3	499.5	-1.0
	0.5 mg/mL	587.8	587.4	-0.1
	0.1 mg/mL	564.6	586.9	4.0
Hemoglobin	1 mg/mL	422.9	400.6	-5.3
	0.5 mg/mL	407.7	406.2	-0.4
	0.1 mg/mL	422.9	453.7	7.3
Biotin	1200 ng/mL	535.1	531.6	-0.7
	600 ng/mL	564.1	521.3	-7.6
Biotin	1200 ng/mL	385.1	397.3	3.2
	600 ng/mL	446.9	436.8	-2.3
Intralipids	20 mg/mL	565.7	551.7	-2.5
	10 mg/mL	582.3	567.6	-2.5
	5 mg/mL	524.2	581.0	10.8
Intralipids	20 mg/mL	442.7	418.8	-5.4
	10 mg/mL	436.1	442.9	1.6
	5 mg/mL	444.5	456.1	2.6
Bilirubin	0.66 mg/mL	453.2	513.5	13.3
	0.2 mg/mL	536.2	573.5	7.0
Bilirubin	0.66 mg/mL	392.7	424.3	8.0
	0.2 mg/mL	440.9	439.2	-0.4

Expected Values:

Expected IGFBP-5 concentrations in rat and mouse samples were calculated by evaluating 10 male and 10 female Sprague Dawley rat samples and 10 male and 10 female Swiss Webster mouse samples in Ansh Labs Total Rat/Mouse IGFBP-5 ELISA. All rat samples were diluted 1:25 and mouse samples were diluted 1:10. IGFBP-5 mean, and median concentrations were calculated using Analyse-It® for Microsoft Excel and is shown below.

Sample	Gender	Strain	No of specimens (n)	Mean (ng/mL)	Median (ng/mL)
Rat	Male	Sprague Dawley	10	1692.2	1598.6
	Female		10	1045.5	1063.4
Mouse	Male	Swiss Webster	10	1346.8	1323.1
	Female		10	1001.3	1046.2

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