# Free IGF-I ELISA

**RUO**

# AL-122

## INTENDED USE

The Free IGF-I enzyme linked immunosorbent assay (ELISA) kit provides materials for the quantitative measurement of Free IGF-I in 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 AND EXPLANATION

Insulin-like growth factor I (IGF-I, a.k.a. somatomedin C) is a 7.6 kDa, 70 amino acid residue peptide, which mediates the actions of growth hormone (GH).[1] IGF-I is synthesized as a prohormone, a polypeptide consisting of A, C, B, D, and E domains.[1, 2] After post-translational modification, the mature IGF-I consist of the A, C, B and D domains, and is structurally homologous to IGF-II and insulin. In vivo, IGF-I is secreted by the liver and several other tissues and is postulated to have mitogenic and metabolic actions at or near the site of synthesis; i.e. paracrine effects.[1] IGF-I also appears in the peripheral circulation where it circulates primarily in a high molecular weight tertiary complex with IGF-binding protein-3 (IGFBP-3) and acid-labile subunit (ALS).[2,3] A smaller proportion of IGF-I circulates in association with other IGF-binding proteins.[3]

Recently, there has been research interest in the measurement of serum/plasma "unbound" IGF-I which, theoretically, is the biologically active fraction. Although the existence of a true unbound IGF serum/plasma compartment is controversial, pharmacokinetic studies indicate that a small percentage of plasma IGF-I is not associated with IGF-binding proteins.[4] Unbound IGF-I has also been observed in saliva.[6] In addition, it appears that IGF-I may exert a tonic hypoglycemic effect under normal conditions that is inhibited by exogenous IGFBP-1 administration.[7]

It is likely that the measured unbound IGF-I fraction is a combination of the true unbound and the fraction of IGF-I that can be readily dissociated from IGFBP's under the specific assay conditions. In this respect, it has been shown that exogenously administered IGF-I almost immediately associates with low MW IGFBP's, then quickly moves into the high MW tertiary complex.[5,8,9] The tertiary complex does not appear to be easily dissociated and does not re-equilibrate with exogenously added IGF-I or IGFBP-3 to a significant degree.[8] On the other hand, the low MW complexes have a rapid turnover, and may be the source for much of the measured unbound IGF-I.

Various methods have been used to estimate the unbound (or freely dissociated) IGF-I fraction.[4,9,10] Size-exclusion chromatography and filtration methods.[4,9] have the theoretical disadvantage of altering the sample matrix and the equilibrium between IGF-I and IGFBP's. A direct detection unbound IGF-I assay using immobilized IGFBP-3 for capture and anti-IGF-I antibody for detection has been reported.[11]

The Ansh Labs Free IGF-I kit uses a highly sensitive two-site antibody method which allows detection of unbound IGF-I. The kit may be used as a "direct" assay to measure the "dissociable" fraction of IGF-I.[8]

## PRINCIPLE OF THE TEST

The Free IGF-I is a quantitative one-step sandwich type immunoassay. In the first step Calibrators, Controls and unknown samples are added to Free IGF-I antibody coated microtiter wells and incubated along with horseradish peroxidase labeled antibody conjugate. After a 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-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 IGF-I in the samples and calibrators.

## MATERIALS SUPPLIED

**CAL-122A** **IGF-I Calibrator A**

One vial, 3.0 mL, labeled IGF-I Calibrator A, containing 0ng/mL IGF-I in buffer with ProClin™ 300. Store unopened at 0 to -20°C until the expiration date. Avoid repeated freeze thaws.

**CAL-121B - CAL-121G** **IGF-I Calibrators B - G**

Six vials, 0.5 mL, labeled B-G, containing concentrations of Human IGF-I in the range of 2.5 to 120ng/mL (Refer to Calibration Card for exact values), in buffer with ProClin™ 300. Store unopened at 0 to -20°C until the expiration date. Avoid repeated freeze thaws.

***NOTE**: The calibrators are traceable to World Health Organization IGF-I preparation NIBSC code 02/254, version 6.0.*

**CTR-121-I & CTR-121-II** **IGF-I Controls I & II**

Two vials, 0.5 mL, labeled Levels I and II containing low and high IGF-I concentrations (Refer to Calibration Card for exact values) in buffer with ProClin™ 300. Store unopened at 0 to -20°C until the expiration date. Avoid repeated freeze thaws.

**PLT-121** **IGF-I Coated Microtitration Strips**

One stripholder, containing 12 strips and 96 microtitration wells with IGF-I 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-121** **IGF-I Enzyme Conjugate Ready-To-Use (RTU)**

One bottle, 12 mL, containing HRP-conjugated IGF-I antibody in buffer with a non-mercury preservative. Store 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 to 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 shaker.
3. Microplate washer.
4. Semi-automated/manual precision pipette to deliver 10–250 µL.
5. Repeater 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

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.

### WARNING: Potential Chemical Hazard

Some reagents in this kit may contain ProClin™ 300 and Sodium azide 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 AND PREPARATION

a) **Serum is the recommended sample type.** Other sample types may yield varying results.

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 should be stored frozen at -80°C or lower immediately upon separation.

d) Avoid repeated freezing and thawing of samples.

e) Avoid assaying lipemic, hemolyzed or icteric samples.

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 Free IGF-I 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

1. **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.

## 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.

Note: Refer the end of this IFU for protocols 1 and 2 in tabular form.

### Protocol-1 (Serum samples)

1. Label the microtitration strips to be used.
2. Pipette **50 µL** each of the **Calibrators, Controls and Unknowns** to the appropriate wells.
   ***Note:*** *In case sensitivity below calibrator B level is desired, dilute calibrator B as below.*
   ***CAL B/2:*** *Mix 50 µL of Calibrator B with 100 µL of Calibrator A in an eppendorf tube.*
3. Add **100 µL** of the **IGF-I Enzyme Conjugate Ready-To-Use (RTU)** to each well using a repeater pipette.
4. 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).
5. Aspirate and wash each strip **5 times** with Wash Solution **(350 µL/per well)** using an automatic microplate washer.
6. Add **100 µL** of the **TMB chromogen solution** to each well using a repeater pipette. Avoid exposure to direct sunlight.
7. Incubate the wells, shaking at **600–800 rpm** on an orbital microplate shaker, for **8-10 minutes** at room temperature (23 ± 2°C).
   ***NOTE****: Visually monitor the color development to optimize the incubation time.*
8. Add **100 µL** of the **Stopping solution** to each well using a repeater pipette. Read the absorbance of the solution in the wells within **10 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**.*

## Protocol-2 (Plasma samples)

Note: EDTA- Plasma samples recover at the low end of the curve and should be validated by the user before use.

1. Label the microtitration strips to be used.
2. Pipette **50 µL** each of the **Calibrators and Controls** to the appropriate wells.
   ***Note:*** *In case sensitivity below calibrator B level is desired, dilute calibrator B as below.*
   ***CAL B/3:*** *Mix 50 µL of Calibrator B with 100 µL of Calibrator A in an eppendorf tube.*
3. Pipette **25 µL** of **samples** using precision pipette to the sample designated wells.
4. Pipette **25 µL** of **IGF-I Calibrator A (CAL-122A)** to the sample added wells only.
   ***NOTE****: Multiply the observed concentrations of samples by dilution factor 2.*
5. Add **100 µL** of the **IGF-I Enzyme Conjugate Ready-To-Use (RTU)** 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 **60 minutes** at room temperature (23 ± 2°C).
7. Aspirate and wash each strip **5 times** with Wash Solution **(350 µL/per well)** using an automatic microplate washer.
8. Add **100 µL** of the **TMB chromogen solution** to each well using a repeater pipette. Avoid exposure to direct sunlight.
9. Incubate the wells, shaking at **600–800 rpm** on an orbital microplate shaker, for **8-10 minutes** at room temperature (23 ± 2°C).
   ***NOTE****: Visually monitor the color development to optimize the incubation time.*
10. Add **100 µL** of the **Stopping solution** to each well using a repeater pipette. Read the absorbance of the solution in the wells within **10 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 IGF-I 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 IGF-I concentrations in ng/mL along the x-axis, using a cubic regression curve-fit.
3. Determine the IGF-I concentrations of the Controls and unknowns from the calibration curve by matching their mean OD readings with the corresponding IGF-I concentrations.
4. Multiply the observed sample concentration by the dilution factor to calculate the total IGF-I concentration in the specimens. For samples diluted as per assay protocol-2, the dilution factor is 2.
5. Any sample reading higher than the highest Calibrator should be reported as such.
6. Any sample reading lower than the analytical sensitivity should be reported as such.

## LIMITATIONS

The reagents supplied in this kit are optimized to measure Free IGF-I 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 heterophilic antibodies in the samples.[14]

## QUALITY CONTROL

- Each laboratory should establish mean values and acceptable ranges to ensure proper performance.
- Free IGF-I ELISA controls or other commercial controls should fall within established confidence limits.
- The confidence limits for Free IGF-I 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 | Calibrators | Mean Absorbance | Conc. (ng/mL) |
|---|---|---|---|
| A1, A2 | A | 0.056 (Blank) | 0 |
| B1, B2 | B | 0.122 | 2.6 |
| C1, C2 | C | 0.331 | 6.2 |
| D1, D2 | D | 0.797 | 13.3 |
| E1, E2 | E | 1.357 | 23.6 |
| F1, F2 | F | 2.430 | 56.0 |
| G1, G2 | G | 3.410 | 120.0 |

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

## ANALYTICAL CHARACTERISTICS

All the concentrations listed are in ng/mL.

**Imprecision:**

Reproducibility of the Free IGF-I assay was determined using two kit controls and two serum pool samples. The study included six assays with samples CI, CII, QC3 and QC5 in replicates.

| Sample | Mean Conc. | Within Run | | Between Run | | Total | |
|---|---|---|---|---|---|---|---|
| ID (n) | (ng/mL) | SD | %CV | SD | %CV | SD | %CV |
| CI (24) | 2.087 | 0.086 | 4.14% | 0.100 | 4.81% | 0.132 | 6.35% |
| CII (23) | 8.191 | 0.350 | 4.27% | 0.341 | 4.17% | 0.489 | 5.97% |
| QC3 (24) | 1.708 | 0.066 | 3.88% | 0.087 | 5.09% | 0.109 | 6.39% |
| QC5 (12) | 4.794 | 0.181 | 3.77% | 0.170 | 3.54% | 0.248 | 5.17% |

**Analytical Sensitivity:**

The analytical sensitivity in the Free IGF-I assay, as calculated by the interpolation of mean plus two standard deviations of 20 replicates of calibrator A and calibrator B, is 0.025 ng/mL.

**Linearity:**

Calibrator G and one serum sample containing various IGF-I levels were diluted with calibrator A. The % recovery on individual samples is represented in the following table.

| Sample ID | Dilution factor | Expected Value (ng/mL) | Observed Value (ng/mL) | %Recovery |
|---|---|---|---|---|
| Calibrator G | Neat | 120.000 | | |
| | 1:2 | 60.000 | 56.235 | 94% |
| | 1:4 | 30.000 | 31.673 | 106% |
| | 1:8 | 15.000 | 15.930 | 106% |

| | | | | |
|---|---|---|---|---|
| | 1:16 | 7.500 | 8.977 | 120% |
| 1 | 1:2 | 36.978 | | |
| | 1:4 | 18.489 | 16.058 | 87% |
| | 1:8 | 9.245 | 7.733 | 84% |
| | 1:16 | 4.622 | 3.960 | 86% |
| | 1:32 | 2.311 | 2.057 | 89% |

### Analytical Specificity:

The monoclonal antibody pair used in the assay detects Free IGF-I. Other related analytes at the concentration in the table below did not show any significant cross-reaction.

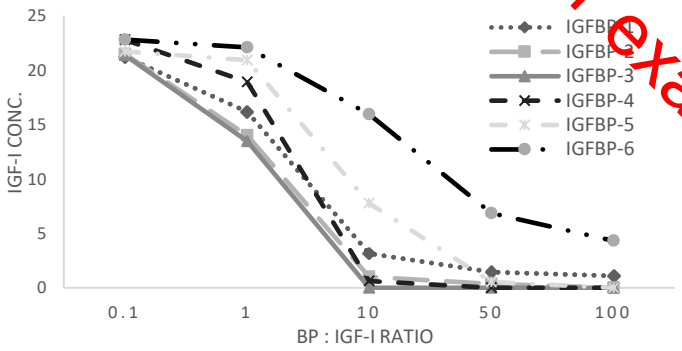
| Sample No. | Cross-reactant | Concentration (ng/mL) | % Cross-reactivity |
|---|---|---|---|
| 1 | IGFBP-2 | 1000 | ND |
| 2 | IGFBP-3 | 1000 | 0.04 |
| 3 | IGFBP-4 | 1000 | ND |
| 4 | IGFBP-5 | 1000 | ND |
| 5 | Rat IGF-I | 1000 | 3.16 |
| 6 | IGF-I/IGFBP-3 complex | 1000 | 0.42 |
| 7 | IGF-II | 1000 | ND |

### Species Reactivity:

This assay detects IGF-I in Bovine, Equine, Canine, Caprine, Ovine, Feline, Porcine, Mouse, Rat, and Rabbit serum samples.

### Inhibition:

Varying molar ratios of IGF-I and IGF-binding proteins 1-6 were mixed together to study the inhibition of IGF-I. Results are outlined in the chart below.




### Interference:

When potential interferents (hemoglobin, triglycerides, and bilirubin) were added at the given concentrations to control sample, Free IGF-I concentration was within ±13% 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 | 1.037 | 1.088 | 4.918 |
| | | 3.308 | 3.537 | 6.923 |
| | | 2.702 | 2.844 | 5.255 |
| Triglycerides | 5 | 1.037 | 1.000 | -3.568 |
| | | 3.308 | 3.394 | 2.600 |
| | | 2.702 | 2.836 | 4.959 |
| Bilirubin | 0.5 | 0.842 | 0.944 | 12.11 |
| | | 3.166 | 3.443 | 8.749 |
| | | 2.984 | 3.125 | 4.725 |

### Expected Values:

The expected ranges for Free IGF-I in pediatric male samples in the age range of 3.0 – 18.0 years were calculated using 95% non-parametric estimation. A total of 398 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 | Number of specimens (n) | Median Conc. (ng/mL) | Free IGF-I (ng/mL) 95% CI |
|---|---|---|---|
| 1 | 213 | 2.9 | 0.8 - 16.7 |
| 2 | 53 | 3.6 | 1.5 - 24.5 |
| 3 | 32 | 4.7 | 1.2 - 10.7 |
| 4 | 50 | 4.6 | 1.7 - 14.6 |
| 5 | 50 | 4.3 | 1.9 - 45.8 |

The expected ranges for Free IGF-I in pediatric female samples in the age range of 2.4 – 18.0 years were calculated using 95% non-parametric estimation. A total of 426 samples in Breast Tanner stages 0 - 5 were evaluated using Analyse-It® for Microsoft Excel as seen in table below.

| Breast Tanner Stage | Number of specimens (n) | Median Conc. (ng/mL) | Free IGF-I (ng/mL) 95% CI |
|---|---|---|---|
| 0 | 14 | 2.8 | 1.7 - 7.7 |
| 1 | 170 | 3.2 | 1.2 - 11.5 |
| 2 | 61 | 4.3 | 1.8 - 31.3 |
| 3 | 58 | 6.9 | 3.0 - 19.7 |
| 4 | 54 | 4.8 | 1.2 - 10.0 |
| 5 | 69 | 3.8 | 1.1 - 14.5 |

***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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Not for use in diagnostic procedures.



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## Free IGF-I ELISA Protocols

| Steps | | Protocol-1 (Serum samples) | Protocol-2 (Plasma samples) |
|---|---|---|---|
| 1. | a. | 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. | |
| | b. | Label Microtitration strips to be used. | |
| | c. | Pipette **50 µL** each of the **Calibrators and Controls** to the appropriate wells. | |
| | d. | Pipette **50 µL** of **samples** using precision pipette to the sample designated wells. | Pipette **25 µL** of **samples** using precision pipette to the sample designated wells. |
| | e. | N/A | Pipette **25 µL** of **IGF-I Calibrator A (CAL-122A)** to the sample added wells only. |
| | f. | Add **100 µL** of the **IGF-I Enzyme Conjugate Ready-To-Use (RTU)** to each well using a repeater pipette. | |
| | g. | 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). | |
| 2. | | Aspirate and wash each strip **5 times** with Wash Solution **(350 µL/per well)** using an automatic microplate washer. | |
| 3. | a. | Add **100 µL** of the **TMB chromogen solution** to each well using a repeater pipette. Avoid exposure to direct sunlight. | |
| | b. | Incubate the wells, shaking at **600–800 rpm** on an orbital microplate shaker, for **8-12 minutes** at room temperature (23 ± 2°C).<br>*NOTE: Visually monitor the color development to optimize the incubation time.* | |
| 4. | | Add **100 µL** of the **Stopping solution** to each well using a repeater pipette. Read the absorbance of the solution in the wells **within 10 minutes**, using a microplate reader set to 450 nm.<br>*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.* | |
| 5. | | N/A | Multiply the observed sample concentration by the **dilution factor of 2** to calculate the total IGF-I concentration in the specimens. |

***NOTE:***

**Protocol-1 and Protocol-2 can also be performed simultaneously in the same run by marking the sample wells as per the protocol used. The sample results then should be processed as per the protocol by applying the dilution factor for protocol-2.**