MEASUREMENT OF IGFBP-1 CONCENTRATIONS IN HUMAN SERUM

Assay Principle:

Serum IGFBP-1 levels are determined using the Total IGFBP-1 IRMA kit manufactured by Diagnostic Systems Laboratories, Inc. (DSL, Webster, TX). The minimum detection limit (as per the manufacturer) is 0.33 ng/ml. The procedure employs a two-site immunoradiometric assay (IRMA). The IRMA is a non-competitive assay in which the analyte to be measured is "sandwiched" between two antibodies. The first antibody is immobilized to the inside wall of the tubes. The other antibody is radiolabelled for detection. The analyte present in the patient samples, standard, and controls is bound by both of the antibodies to form a "sandwich" complex. Unbound materials are removed by decanting and washing the tubes. The radioactivity is measured in a gamma counter. A standard curve based on the concurrent testing of the calibrators is generated and sample concentrations of IGFBP-1 are extrapolated from the curve.

Assay Procedure:

Serum should be used. Plasma is not recommended for the measurement of IGFBP-1.

Human control samples (I and II) provided by DSL and an in-house human control (QCV) are prepared simultaneously to verify the effectiveness of the assay.

Creation of the assay standard curve is achieved using standards (B-G) of known concentrations (provided by DSL).

Standards, controls and unknowns (25 μl) are pipetted into duplicate antibody-coated tubes.

Assay buffer (200 μl) is added to each tube.

Samples are vortexed for 1-2 seconds and allowed to incubate at room temperature (~ 25°C) for 4 hours on a shaker set at 180 rpm.

MEASUREMENT OF IGFBP-1 CONCENTRATIONS IN HUMAN SERUM

Assay Procedure (continued):

Tubes are decanted and washed with 2 mL of deionized water for a total of two washings.

Anti-IGFBP-1 [I-125] reagent is added to the tubes and allowed to incubate at room temperature on a shaker set at 180 rpm for 18-22 hours.

Tubes are decanted and washed with 2 mL of wash solution for a total of three washings.

The activity of the tubes is measured for one minute using a Packard RIAStar Gamma Counter model C5410.

A standard curve is constructed (using software provided by Packard Instruments) and the concentrations of the unknowns and controls read from this curve.

The IGFBP-1 data is transferred to a Power Macintosh 6500/250 and set-up as a Microsoft Excel spreadsheet.

ACTIVE™ Total IGFBP-I IRMA

DSL-7800

Revision date: May 8, 2001

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I. INTENDED USE

The DSL-7800 ACTIVE™ Total IGFBP-1 Coated-Tube Immunoradiometric Assay Kit provides materials for the quantitative measurement of total IGFBP-1 in serum.

> NORTH AMERICAN CUSTOMERS ONLY:
> This assay is intended for research use only and not for use in diagnostic procedures. For use only by researchers and laboratories certified by Diagnostic Systems Laboratories, Inc., 445 Medical Center Blvd., Webster, TX 77598.

II. SUMMARY AND EXPLANATION OF THE TEST

Insulin-like Growth Factor Binding Protein-1 (IGFBP-1) is one of six structurally related proteins which specifically binds and modulates the bioactivity of the mitogenic peptides, IGF-I and IGF-II [1]. IGFBP-1 is a 25.3 kDa phosphoprotein synthesized primarily in liver and decidualized uterine endometrium; low levels of synthesis may also occur in luteal-phase granulosa cells and in non-human primate and rodent kidney [1-5]. IGFBP-1 is distributed primarily in the intravascular space and lymph, and low quantities have been detected in urine [1]. *In vitro*, IGFBP-1 synthesis has been identified in continuous human cell lines derived from liver (HepG2), breast (MDA-MB-231 and HS578t) and uterine endometrium (HEC1B and KLE) [1,2,6,7].

IGFBP-1 levels are high in amniotic fluid and fetal serum, and serum levels decline with age during postnatal life [1,8]. Elevated IGFBP-1 serum levels have been identified in pregnancy, renal failure, malnutrition, insulin-deficiency (e.g. insulin-dependent diabetes mellitus) and liver cirrhosis and carcinoma [1]. Low IGFBP-1 levels are found in obesity and other hyperinsulinemic states [1,9]. IGFBP-1 levels are relatively elevated in intrauterine growth retardation and low in large-for-gestational-age infants [1,10].

Serum IGFBP-1 levels in normal individuals and in individuals with growth hormone deficiency and other disorders vary considerably in relation to meals. Levels increase 2- to >20-fold after an overnight fast and show an exponential fall following a meal [1,11]. Studies indicate that these changes are inversely related to insulin concentrations and are not correlated with growth hormone or glucose levels [11,12].

IGFBP-1 undergoes rapid *in vivo* metabolism and clearance, with a $T_{1/2}$ estimated at 8-12 minutes [13]. Degradation of IGFBP-1 mRNA is also rapid and IGFBP-1 mRNA transcription is potently suppressed by insulin *in vitro* [14]. These regulatory dynamics probably account for the rapid fall in serum IGFBP-1 levels following a meal. The site of IGFBP-1 clearance has not been identified, and degradation products have not been identified in serum. Plasma components, including plasmin [15], may play a role in IGFBP-1 degradation.

Insulin suppression of IGFBP-1 appears to be dominant over the stimulatory effects of glucocorticoids, cAMP, progesterone and, possibly, glucagon and somatostatin [16-18]. *In vitro*, phorbol esters stimulate IGFBP-1 production apparently through secondary down-regulation of protein-kinase C; this effect appears to dominate over the suppressive effects of insulin [19]. Increased IGFBP-1 synthesis has also been observed during hepatic regeneration, the mechanism of which is not yet known [1].

Although its physiologic role has not been completely defined, the nature of its regulation suggests that IGFBP-1 may be involved in glucose counter-regulation and IGF-mediated substrate utilization [1,20]. IGFBP-1 has been shown to inhibit IGF-mediated mitogenesis *in vitro* and IGF-induced hypoglycemia in rodents [1,13]. However, a number of reports have shown that IGFBP-1 is capable of both inhibition [13, 22] as well as augmentation [23,24] of IGF bioactivity. These apparently opposing observations may be explained by the recent findings that differential phosphorylation of IGFBP-1 could significantly alter its affinity for IGFs and, therefore, its capacity to modulate their cellular responsiveness. As many as five IGFBP-1 variants, differing in their state of phosphorylation and relative expression in serum, amniotic fluid, decidua and cell culture media have been reported [25-29]. It has been demonstrated that non-phosphorylated IGFBP-1 has 4- to 6-fold lower affinity for IGF-I compared with phosphorylated IGFBP-1 variants, with phosphorylation occurring only on serine residues 101, 119 and 169 [25, 27].

Measurement of IGFBP-1 levels in serum and cell-conditioned medium may be useful as a marker of *in vivo* and *in vitro* insulin action and hepatocyte function for research investigations. In addition, further definition of the physiological functions of IGFBP-1 and the molecular mechanisms by which IGFBP-1 phosphorylation regulates IGF actions would ultimately depend on reliable quantification of its total (absolute) levels. However, because altered phosphorylation results in altered IGFBP-1 immunoreactivity [29, 30], immunoassays which do not detect all of the phosphorylation variants of IGFBP-1 may significantly underestimate IGFBP-1 levels.

The DSL Total IGFBP-1 IRMA was designed to eliminate differential antibody recognition of the various IGFBP-1 phosphoforms. The assay is unaffected by the state of IGFBP-1 phosphorylation, thus, permitting reliable measurement of total IGFBP-1 levels in serum or other biological material. Availability of the DSL Total IGFBP-1 IRMA should greatly facilitate research and clinical investigations of this interesting IGF binding protein. Furthermore, the DSL IGFBP-1 IRMA avoids cross-reactivity to other IGFBPs and is specific for human and primate IGFBP-1, facilitating studies of human IGFBP-1 administration in non-primate systems.

III. PRINCIPLE OF THE TEST

The procedure employs a two-site immunoradiometric assay (IRMA) principle described by Miles et al [21]. The IRMA is a non-competitive assay in which the analyte to be measured is "sandwiched" between two antibodies. The first antibody is immobilized to the inside walls of the tubes. The other antibody is radiolabeled for detection. The analyte present in the unknown, Standards and controls is bound by both of the antibodies to form a "sandwich" complex. Unbound materials are removed by decanting and washing the tubes.

IV. REAGENTS

The DSL-7800 ACTIVE™ Total IGFBP-1 Coated-Tube IRMA Kit contains sufficient reagents for 100 tubes. Each kit contains the following reagents:

A. IGFBP-1 Sample Diluent/Standard A:
One vial, labeled A, 11 mL, containing 0 ng/mL IGFBP-1 in non-human serum with sodium azide as a preservative. Store at 2-8 °C for up to 2 weeks. For longer periods store at -20 °C or lower until expiration date.

B. IGFBP-1 Standards: (Lyophilized)
Six vials, labeled B-G, containing concentrations of 0.5, 2.0, 10.0, 40.0, 80.0 and 160.0 ng/mL IGFBP-1 in non-human serum with sodium azide as a preservative. Refer to vial labels for exact concentrations. Store unopened at 2-8°C until expiration date. Reconstitute each vial with 1 mL deionized water. Upon reconstitution, store at 2-8°C for up to 3 weeks. For longer periods, store at -20°C or lower until expiration date.

The DSL IGFBP-1 IRMA Standards have been calibrated against pure human IGFBP-1 from amniotic fluid [2] and recombinant DNA-derived human IGFBP-1.

C. Anti-IGFBP-1 [I-125] Reagent: (YELLOW)
One bottle, 22 mL, containing < 10 µCi (370 kBq) of [I-125]-labeled anti-IGFBP-1 antibody in a protein-based buffer with sodium azide as a preservative. Store at 2-8°C until expiration date.

D. Anti-IGFBP-1-Coated Tubes: (Yellow Rim)
One hundred (100) plastic tubes with anti-IGFBP-1 antibody immobilized to the inside wall of each tube. Store tubes at 2-30°C until expiration date.

E. IGFBP-1 Controls: (Lyophilized)
Two vials, Levels I and II, containing low and high concentrations of IGFBP-1 in non-human serum with sodium azide as a preservative. Refer to vial labels for control ranges. Store unopened at 2-8°C until expiration date. Reconstitute each vial with 1 mL deionized water. Upon reconstitution, store at 2-8°C for up to 3 weeks. For longer periods, store at -20°C or lower until expiration date.

F. Assay Buffer G: (Green)
One bottle, 22 mL, containing a protein-based buffer with a non-mercury preservative. Store at 2-8° C until expiration date.

G. Wash Concentrate: 70 mL + 630 mL WTR
One bottle, 70 mL, containing wash concentrate with sodium azide as a preservative. Dilute 1:10 with deionized water prior to use. Store at room temperature (~25° C) until expiration date.

NOTE: *All reagents and samples must be allowed to reach room temperature (~25 °C) and mixed thoroughly by gentle inversion before use.*

V. PRECAUTIONS

For in vitro use.

Radioactive Material - Not for Internal or External Use in Humans or Animals.

This radioactive material may be received, acquired, possessed, and used only by physicians, clinical laboratories or hospitals and only for *in vitro* clinical or laboratory tests not involving internal or external administration of the material, or the radiation therefrom to human beings or animals. Its receipt, acquisition, possession, use and transfer are subject to the regulations of each country.

The following precautions should be observed in handling radioactive material:

- Store radioactive materials in a designated area.
- Do not eat, drink, smoke or apply cosmetics where radioactive materials are being handled.
- Do not pipet by mouth.
- Wear gloves when handling radioactive materials and wash hands thoroughly afterwards.
- Cover working area with disposable absorbent paper.
- Wipe up all spills immediately and thoroughly and dispose of the contaminated materials as radioactive waste.
- Dispose of the liquid radioactive waste into the sanitary sewage system if permitted by the local regulations.

WARNING: POTENTIAL BIOHAZARDOUS MATERIAL
This kit may contain some reagents made with human serum or plasma. The serum or plasma used has been tested by an FDA-approved method and found to be non-reactive for HIV-1/2 Antibodies, HCV and HBsAg. Because no method can offer complete assurance that HIV-1/2, HCV, HBsAg, or other infectious agents are absent, these reagents should be handled at the Biosafety Level 2 as recommended for any potentially infectious human serum or blood specimen in the Centers for Disease Control/National Institutes of Health manual "Biosafety in Microbiological and Biomedical Laboratories," 3rd Edition, 1993.

CHEMICAL HAZARD:
As stated in Section IV, some of the reagents in this kit contain sodium azide as a preservative. For all such reagents, the concentration of sodium azide is ≤ 0.09%. Sodium azide may react with lead and copper plumbing to form explosive metal azides. Dispose of all nonradioactive reagents by flushing with large amounts of water through the plumbing system.

VI. SPECIMEN COLLECTION AND PREPARATION

Serum should be used and the usual precautions for venipuncture should be observed. Specimens may be stored at 2-8°C for up to 24 hours and frozen at -20°C or lower for up to 6 months. Avoid repeated freezing and thawing of samples.

Do not use grossly hemolysed or lipemic specimens. Frozen samples should be thawed and mixed thoroughly by gentle swirling or inversion prior to use.

Samples containing IGFBP-1 >160 ng/mL should be diluted with the IGFBP-1 Sample Diluent (Standard A) prior to assay. Each laboratory should determine appropriate dilution factors for the samples to be measured.

The two-step immunoassay format of the DSL Total IGFBP-1 IRMA eliminates the high dose hook effect often associated with the one-step sandwich-type immunoassays [31]. However, samples which give measured IGFBP-1 levels at or above the highest standard should be diluted with the Sample Diluent (Standard A) and re-assayed.

VII. PROCEDURAL NOTES

A thorough understanding of this package insert is necessary for successful use of the product. Reliable results will only be obtained by using precise laboratory techniques and accurately following the package insert. A standard curve must be included with each assay.

Do not mix various lots of any kit component within an individual assay. Do not use any component beyond the expiration date shown on its label.

After removing assay reagents from the refrigerator, allow them to reach room temperature (~25°C) before pipetting. Unused reagents should be stored as specified in the Reagent Section. Standards and Controls should be mixed before use by inverting or swirling gently rather than vortexing. To insure a homogeneous mixture of the reagents in each assay tube, gentle and thorough shaking or vortexing is essential.

Failure to obtain the appropriate values for controls may indicate imprecise manipulation, improper sample handling or deterioration of reagents. Failure to blot tubes adequately following decantation may result in poor replication and spurious values.

VIII. TEST PROCEDURE

A. Materials Supplied:
Materials supplied in the DSL ACTIVE™ Total IGFBP-1 Coated-Tube IRMA Kit, Catalog No. DSL-7800:

MATERIAL	QUANTITY	CATALOG #
IGFBP-1 Standard A	One Vial	7801
IGFBP-1 Standard B	One Vial	7802
IGFBP-1 Standard C	One Vial	7803
IGFBP-1 Standard D	One Vial	7804
IGFBP-1 Standard E	One Vial	7805
IGFBP-1 Standard F	One Vial	7806
IGFBP-1 Standard G	One Vial	7807
IGFBP-1 Control Level I	One Vial	7851
IGFBP-1 Control Level II	One Vial	7852
Anti-IGFBP-1-Coated Tubes	100 Tubes	7210
Anti-IGFBP-1 [I-125] Reagent	One Bottle	7820
Assay Buffer G	One Bottle	10-7840
Wash Concentrate H	One Bottle	5140

B. Materials Required But Not Supplied:

- 12 x 75 mm plastic test tubes
- Test tube rack for 12 x 75 mm tubes
- Precision pipette to deliver 25 µL
- Repeating pipette to deliver 200 µL and 2-3 mL
- Vortex mixer
- A sponge rack or similar device for decantation
- Deionized water
- Shaker capable of 180 rpm
- Absorbent material for blotting tubes
- Gamma counter
- Log-log graph paper or computer IRMA data analysis program

IGFBP-1

C. Preparation of Reagents:

Wash Solution:
Dilute Wash Concentrate 1:10 with deionized water prior to use.

D. Assay Procedure:

Allow all reagents to reach room temperature (~25°C) and mix thoroughly by gentle inversion before use. After reconstitution of reagents, mix thoroughly, avoiding foam. Assay Standards, Controls and unknowns in duplicate.

1. Label two plain (uncoated) tubes for Total Counts. Label and arrange Anti-IGFBP-1-Coated Tubes in duplicate for Standards, Controls and unknowns.
2. Pipet 25 µL of the Standards, Controls or unknowns to the bottom of the appropriate tubes.
3. Add 200 µL of the Assay Buffer to each tube except the Total Count tubes.
4. Vortex gently for 1-2 seconds. Incubate all tubes at room temperature (~25°C) for 4 hours on a shaker set at 180 rpm.
5. Aspirate or decant all tubes, except Total Count tubes, by simultaneous inversion with a sponge rack into a suitable waste receptacle. Strike the tubes sharply on absorbent material to facilitate complete drainage. Blot the tubes to remove any droplets adhering to the rim before returning them to the upright position. Failure to blot tubes adequately may result in poor replication and spurious values.
6. Add 2 mL of deionized water to all tubes except Total Counts tubes. Decant into a waste receptacle. Repeat for a total of 2 washings.
7. Add 200 µL of the Anti-IGFBP-1 [I-125] Reagent to all tubes.
8. Incubate all tubes at room temperature (~25° C) on a shaker set at 180 rpm for 18-22 hours.
9. Aspirate or decant all tubes, except Total Count tubes, by simultaneous inversion with a sponge rack into a radioactive waste receptacle. Strike the tubes sharply on absorbent material to facilitate complete drainage and then allow them to drain on absorbent material for 1-2 minutes. Blot the tubes to remove any droplets adhering to the rim before returning them to the upright position. Failure to blot tubes adequately may result in poor replication and spurious values.
10. Wash all tubes, except Total Counts tubes, by adding 2 mL of Wash Solution. Decant per step 9 twice and repeat wash step twice for a total of three washings.
11. Count all tubes in a gamma counter for one minute.

IX. RESULTS

The results in this package insert were calculated using a log-log curve fit. Other data reduction methods may give slightly different results.

A. Calculate the net counts per minute (net cpm) by subtracting the mean counts per minute (mean cpm) of the 0 ng/mL Standard from the mean cpm of each Standard, Control and unknown. Calculate the % B/T for each Standard, Control and unknown as follows:

$$\% B/T = \frac{\text{Net CPM}}{\text{Mean Total CPM}} \times 100$$

B. Plot a curve of the net cpm or % B/T for each Standard (y-axis) against the IGFBP-1 concentration (x-axis) on log-log graph paper. Draw a standard curve through the mean of the duplicate points.

C. Based on the net cpm or % B/T for each Control and unknown, determine the IGFBP-1 concentration of the means of the duplicate counts of each Control and unknown from the standard curve.

D. Any sample reading greater than the highest Standard should be diluted appropriately with the 0 ng/mL IGFBP-1 Standard and reassayed.

E. Any sample reading lower than the lowest Standard should be reported as such.

X. LIMITATIONS

- The reagents supplied in this kit are optimized to measure IGFBP-1 levels in serum.
- Avoid repeated freezing and thawing of reagents or samples.
- Hemolyzed, icteric and lipemic specimens may give false values and should be avoided.

XI. QUALITY CONTROL

- DSL Controls or other commercial controls should fall within established confidence limits. The confidence limits for DSL Controls are printed on the Control vial labels.
- Low and high level controls should be included in each assay.

TYPICAL ACTIVE™ TOTAL IGFBP-1 IRMA STANDARD CURVE DATA

TUBE NO.	TUBE LABEL	BOUND (cpm)	MEAN (cpm)	B/T (%)	IGFBP-1 (ng/mL)
1, 2	TOTAL COUNTS	153498 152030	152764		
	STANDARDS				
3, 4	A	231 246	239		0.00
5, 6	B	628 599	614	0.3	0.78
7, 8	C	1299 1289	1294	0.7	2.50
9, 10	D	7067 7379	7223	4.6	12.00
11, 12	E	25703 23538	24620	16.0	40.00
13, 14	F	41849 41528	41689	27.1	80.00
15, 16	G	61530 63243	62386	40.7	160.00
	CONTROLS				
17, 18	Level I	1389 1439	1414	0.6	2.73
19, 20	Level II	6972 7678	7325	4.6	12.17

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

XII. EXPECTED VALUES

Each laboratory should establish its own normal ranges.

XIII. PERFORMANCE CHARACTERISTICS

A. Sensitivity:

The theoretical sensitivity, or minimum detection limit, calculated by the interpolation of the mean plus two standard deviations of 20 replicates of the 0 ng/mL IGFBP-1 Standard, is 0.33 ng/mL.

B. Precision:

The intra-assay precision was determined from the mean of 14 replicates each.

SAMPLE	N	MEAN (ng/mL)	STANDARD DEVIATION (ng/mL)	COEFFICIENT OF VARIATION (%)
I	14	5.23	0.27	5.2
II	14	50.28	2.32	4.6
III	14	144.60	3.89	2.7

The inter-assay precision was determined from the mean of average duplicates for 6 separate runs.

SAMPLE	N	MEAN (ng/mL)	STANDARD DEVIATION (ng/mL)	COEFFICIENT OF VARIATION (%)
I	6	5.16	0.18	3.5
II	6	47.08	2.84	6.0
III	6	142.03	5.13	3.6

C. Recovery:

Two serum samples containing different levels of endogenous IGFBP-1 were spiked with known amounts of IGFBP-1 and assayed.

SAMPLE	ENDOGENOUS (ng/mL)	ADDED (ng/mL)	EXPECTED (ng/mL)	OBSERVED (ng/mL)	RECOVERY (%)
I	5.15	5.0	10.15	10.35	102
		60.0	65.15	70.79	109
		100.0	105.15	95.37	91
II	35.40	5.0	40.40	39.93	99
		60.0	95.40	99.18	104
		100.0	135.40	149.08	110

D. Linearity of Dilution:

Three serum samples were diluted with 0 ng/mL IGFBP-1 Standard and assayed.

SAMPLE	DILUTION FACTOR	EXPECTED (ng/mL)	OBSERVED (ng/mL)	RECOVERY (%)
I	---	---	2.74	---
	1:2	1.37	1.20	88
	1:4	0.69	0.70	101
II	---	---	33.50	---
	1:2	16.75	16.31	97
	1:4	8.38	8.03	96
	1:8	4.19	4.11	98
	1:16	2.09	1.95	93
III	---	---	141.50	---
	1:2	70.75	63.44	90
	1:4	35.38	30.12	85
	1:8	17.69	15.07	85
	1:16	8.84	7.44	84
	1:32	4.42	3.58	81
	1:64	2.21	2.18	99

E. Specificity:

The cross-reactivity of the IGFBP-1 antibody has been measured against the following compounds with the DSL-7800 ACTIVE™ Total IGFBP-1 Coated-Tube IRMA.

COMPOUND	% CROSS-REACTIVITY
IGFBP-1	100
IGFBP-2 (5 µg/mL)	ND
IGFBP-3 Glycosylated (5 µg/mL)	ND
IGFBP-3 Non-Glycosylated (5 µg/mL)	ND
IGFBP-4 (1 µg/mL)	ND
IGFBP-5 (2 µg/mL)	ND
IGFBP-6 (1 µg/mL)	ND

ND = *Non-Detectable at the indicated concentrations.*

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