biosensis® Human Insulin-like growth factor-binding protein 1/IGFBP-1/IBP-1 ELISA

Kit Protocol

Catalog No: BEK-2030-1P

For quantitative detection of human IGFBP-1 in human cell culture supernatants, serum, urine, and EDTA, or heparin treated plasma samples only when used as directed.

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I. Materials Provided

Kit Components	Unit Size/Volume
Lyophilized recombinant human IGFBP-1 standard	10ng/tube x 2
96 wells plate pre-coated with anti-human IGFBP-1 antibody	1 plate
Sample diluent buffer	30 ml x1
Biotinylated anti- human IGFBP-1 antibody (dilution 1:100)	130 µl x 1
Antibody diluent buffer	12 ml x 1
Avidin-Biotin-Peroxidase Complex (ABC) (dilution 1:100)	130 µl x 1
ABC diluent buffer	12 ml x 1
TMB colour developing agent	10 ml x 1
TMB stop solution	10 ml x 1

WARNING: DO NOT MIX REAGENTS SUCH AS ANTIBODY BUFFERS ETC BETWEEN DIFFERENT ASSAYS IF USING MULTIPLE KITS. USE ONLY THE BUFFERS, STANDARDS & SOLUTIONS INCLUDED FOR YOUR SPECIFIC LOT. MIXING SOLUTIONS FROM DIFFERENT TARGET KITS CAN LEAD TO POOR KIT PERFORMANCE

II. Equipment Required but Not Supplied

1. Wash buffer (0.01M PBS or TBS). *See technical hints*
2. Automated plate washer is desirable
3. Multi-channel or repeating pipette.
4. Clean tubes and Eppendorf tubes.
5. Plate shaker (300-500 rpm)
6. Mechanical Vortex.
7. Microplate reader with 450nm filter.

III. Technical Hints

1. Spin all kit components to get liquid to the bottom of tubes before use.

2. Duplicate well assays are recommended for both standard and sample testing.

3. In order to avoid marginal effects of plate incubation due to temperature differences, it is suggested that the ABC and TMB solutions be brought to room temperature (or 37° C if using option protocol) 30 min before use.

4. Recommended method for manual plate washing.

- Emptying of wells on the plate:
  Place the plate on the palm of the hand in a position that enables easy flicking movement using the wrist. Holding the plate over a sink, quickly invert the plate, whilst accelerating the arm downward toward the sink. Abruptly stop the downward acceleration to force the liquid from the wells into the sink. When done correctly the technique should prevent liquid from getting on to the fingers or on the outside of the strip wells or plate holder. *Note: Retain the upside down position of the plate to avoid any back flow into the wells. DO NOT LET THE PLATE DRY OUT.*

- Blotting the plate:
  Immediately blot the inverted plate by lightly tapping the plate 3-4 times on blotting paper.

- Washing: Forcefully pipette Wash Buffer into each well with a multi-channel pipette. Empty the wells of wash buffer using technique described above. Repeat washing and flicking procedures thrice. Wash plate 0.01M TBS or 0.01M PBS, and each time let washing buffer stay in the wells for 1-2 min before rinsing with more wash buffer as directed above. *Note: **Avoid** touching the inside surface of the wells with the pipette tips.*

Do not let the wells dry out at any time or enzymatic activity will be lost.

5. Preparation of Wash buffer: (not provided in the kit)

Preparation of 0.01M **TBS:** Add 1.2g Tris, 8.5g Nacl; 450µl of purified acetic acid or 700µl of concentrated hydrochloric acid to 1000ml H_2O and adjust pH to 7.2-7.6. Finally, adjust the total volume to 1L.

Preparation of 0.01 M **PBS:** Add 8.5g sodium chloride, 1.4g Na_2HPO_4 and 0.2g NaH_2PO_4 to 1000ml distilled water and adjust pH to 7.2-7.6. Finally, adjust the total volume to 1L.

Note: PBS buffers typically work more reliably than TBS buffers in most cases are recommended.

Note: if blank values are over 0.2 the addition of 0.05% Tween-20 to either TBS or PBS wash buffers will usually help lower them as well as overall assay backgrounds and can help improve signal/noise ratios.

IV. Storage of Kit Components

This kit may be stored for up to 6 months at 2°C to 8°C from the date of shipment. Standards should be stored at -20°C or -80°C (recommended at –80°C) after reconstitution. Coated strips or reagents may be store for up to 1 month at 2°C to 8°C. Return unused wells to the pack and reseal pack. *Note: the kit can be used within 8 months if the whole kit is stored at -20°C. Avoid repeated freeze-thaw cycles.*

V. Sample Preparation and Dilution

- **Cell culture supernatant:** Remove particulates by centrifugation (10,000 x g 5 min), analyze immediately or aliquot and store at -20°C-80°C. Divide samples into aliquots to prevent multiple freeze thaws; use appropriate protease inhibitor cocktails for best results.

- **Serum**: Allow the serum to clot in a serum separator tube (about 4 hours) at room temperature or coat at 4°C overnight. Centrifuge at approximately 2000 X g for 15 min. Analyze the serum immediately or aliquot and store frozen at -20°C.

- **Plasma**: Collect plasma using **heparin,** or **EDTA** as an anticoagulant. Centrifuge for 15 min at 1500 x g within 30 min of collection. Assay immediately or aliquot and store samples at -20°C-80°C.

- **Urine**: Aseptically collect. Remove particular impurities and precipitates by centrifugation, and/or particle removal via syringe or another filtration device. Solution must be particle and cell free for best results. Assay immediately or aliquot and store samples at -20°C to -80°C. Note: treatment of urine with Thimerosal or other proteinase inhibitors can increase signal yields in ELISA assays for some targets, please see: (Hoyer, J. et al. (1999). "Effects of microgravity on urinary osteopontin". J. Am. Soc. Nephrol. **10**: S389-S393. Note 2: Urine can be quite acidic; neutralize with mild base the final assay sample to pH 7.0-7.5 for best results.

Note: IGFBP-1 is bound to protein ligands in serum and plasma samples, and to a lesser extent in all samples, an optional acid pretreatment step to release IGFBP1 as free for is recommended for the best results. Pretreatment is via an acidification/neutralization step prior to using the samples in the ELISA assay. It is not necessary to pretreat the recombinant standard included in this ELISA, as it is free of inactivating binding proteins already. Please see Appendix A for pretreatment steps.

Note: The use of proteinase inhibitors such as Aprotinin is recommended for or ALL sample collection methods to prevent sample degradation. A suggested amount is 0.5 TIU Trypsin Inhibitor Unit (TIU) per ml of sample solution.

Note: The user needs to estimate the concentration of the target protein in the sample and select a proper dilution factor so that the diluted target protein concentration falls near the middle of the linear regime in the standard curve. Also with certain growth factors and target proteins, binding proteins and location can influence over all signal. It is recommended that researchers confirm by literature an effective lysis, cell membrane preparations and/or extraction buffer formulations and treatments that are suitable for the precise target being assayed.

The following table can be used as a guideline for sample dilution. Dilute the sample using the provided diluent buffer. Mix the sample thoroughly with diluent buffer.

Target protein concentration	**Concentration range**	**Working dilution**	**Amount of sample diluent buffer to be added**
High target protein concentration	**20- 200 ng/ml**	1:100	Add 1 µl sample into 99 µl sample diluent buffer
Medium target protein concentration	**2-20 ng/ml**	1:10	Add 10 µl sample into 90 µl sample diluent buffer
Low target protein concentration	**31.2-2000 pg/ml**	1:2	Add 50 µl sample to 50 µl sample diluent buffer
Very Low target protein concentration	**≤31.2 pg/ml**	No dilution necessary	(working dilution is 1:2)

Assay Precision:

	Intra-Assay Precision			**Inter-Assay Precision**		
Sample	1	2	3	1	2	3
N	16	16	16	24	24	24
Mean (pg/ml)	145	736	1243	173	687	1360
Stan. Dev.	5.09	37.54	70.85	10.21	42.02	102
CV(%)	4.2	5.1	5.7	5.9	6.3	7.5

Range 31.2 pg/ml-2000pg/ml
Sensitivity < 1 pg/ml
Specificity: Natural and recombinant forms of human IGFBP-1.
Cross-reactivity No detectable cross-reactivity with other relevant proteins

VI. Reagent Preparation

1. Preparation of Standards

- Reconstitution of standard with the sample diluent provided.

  (a) 10,000 pg/ml (10 ng/ml) of human IGFBP-1 standard solution: Add 1ml of sample diluent buffer into ***one*** tube of standard, mix thoroughly and keep the tube at room temperature for 10 min. The standard product used in this kit is recombinant human IGFBP-1 amino acids 26-272 produced in and purified from a mammalian cell expression system.

  (b) 2000 pg/ml of human IGFBP-1 standard solution: Add 0.2 ml of the above 10 ng/ml IGFBP-1 standard solution into 0.8 ml sample diluent buffer and mix thoroughly.

  (c) 1000 pg/ml to 31.2 pg/ml of human IGFBP-1 standard solutions: Label 6 Eppendorf tubes with 1000 pg/mL, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.2 pg/ml, respectively. Aliquot 0.3 ml of sample diluent buffer into each tube. THEN Add 0.3 ml of the above 2000 pg/ml standard solution (item b) into the 1st tube and mix. Transfer 0.3ml from 1st tube into 2nd and mix. Continue performing serial dilutions.

- *Note: Standard solution should be prepared no more than 2 hours prior to the experiment. The working standard solution may be stored at 4°C for up to 12 hours, or at -20°C for up to 48 hours. Avoid repeated freeze-thaw cycles.*

2. Preparation of Biotinylated antibody working solution

- The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume).
- Biotinylated antibody (Reagent B) should be diluted in 1:99 with the antibody diluent buffer and mixed thoroughly.
- *Note: the solution should be prepared no more than two hours prior to the experiment.*

3. Avidin-Biotin-Peroxidase Complex (ABC)

- The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume).
- ABC should be diluted in 1:99 with the ABC diluent buffer and mixed thoroughly.
- *Note: the solution should be prepared no more than one hour prior to the experiment.*

VII. Assay Procedure

We recommend that standard solution dilutions and each sample are plated in duplicate. Read entire protocol before beginning; if using optional method the ABC working solution and TMB color developing agent must be kept warm at 37°C for 30 min before use.

1. Aliquot out the standards into each well of the pre-coated plate. Add 0.1ml of the sample diluent buffer into the control well (Zero well). Add 0.1ml of each properly diluted sample into each empty well. We recommend at least duplicate standards and samples. *Note: See "VII. Sample Preparation and Dilution Guideline" above for details*.
2. Seal the plate with parafilm and incubate the plate at 4°C overnight. *Note: we recommend users leave the plate on a shaker if possible*. (Optional method: 37°C, 90 minutes but it is slightly less sensitive)
3. Remove the cover, discard the plate contents and blot the plate onto paper towels or other absorbent material. **DO NOT** let the wells completely dry at any time. Wash plate 3-5 times using wash buffer as described in the technical hints. *Note: See "V. Technical Hints - Recommended method for manual plate washing" above for details*.
4. Add 0.1ml of biotinylated antibody working solution into each well. Cover and incubate the plate at room temperature on a plate shaker for 2-3 hours. (Optional 37°C, 60 minutes with shaking)Wash 5X as described in the technical hints.
5. Add 0.1ml of prepared ABC working solution (*Note: See "VIII. Reagent Preparation"* above for details) into each well. Cover and incubate the plate at room temperature on a plate shaker for 1 hour. (Optional 37°C, 30 minutes with shaking). Wash 5X as described in technical hints allowing the wash buffer to remain in the wells for 1-2 minutes.
6. Warm TMB to room temperature (37° if using optional protocol). Add 90µl of prepared TMB color developing agent into each well and incubate plate at room temperature for 5-30 min in the dark (shades of blue can be seen in the wells with the four most concentrated standard solutions; the other wells show no obvious color).
7. Stop the reaction by adding 0.1ml of prepared TMB stop solution into each well. The color will change into yellow.
8. Immediately read the plate at 450nm with a microplate reader. *Note: Color will fade over time; hence, we recommend plate to be read within 30 min after adding the stop solution*.

CAUTION: Bubbles in the wells will cause inaccurate readings. Ensure that all bubbles are removed prior to taking the absorbance reading.

VIII. Calculation of Results

(a) Manual Plate Reading:

(The relative $O.D._{.450}$) = (the $O.D._{.450}$ of each well) – (the $O.D._{.450}$ of Zero well).

The standard curve can be plotted as the relative $O.D._{.450}$ of each standard solution (Y) vs. the respective concentration of the standard solution (X). Known concentrations of the target protein are plotted on the X-axis and the corresponding $O.D._{.450}$ on the Y-axis. The standard curve should result in a graph that shows a direct relationship between target protein concentrations and the corresponding $O.D._{.450.}$ The greater the concentration of target protein in the sample, the higher the $O.D._{.450.}$

Note: if the samples measured were diluted, multiply the dilution factor to the concentrations from interpolation to obtain the concentration before dilution.

Determine concentration of target protein in unknown sample:

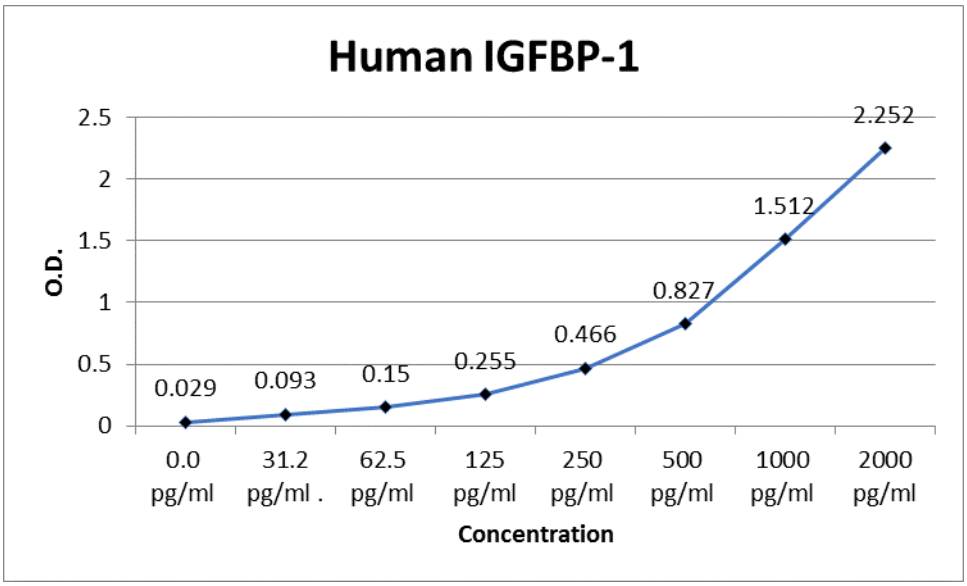
The target protein concentration of the samples can be interpolated from the standard curve. Draw a horizontal line to intersect with the standard curve. A vertical line dropped from this point intersects the X-axis at the concentration of antibody in the unknown sample.

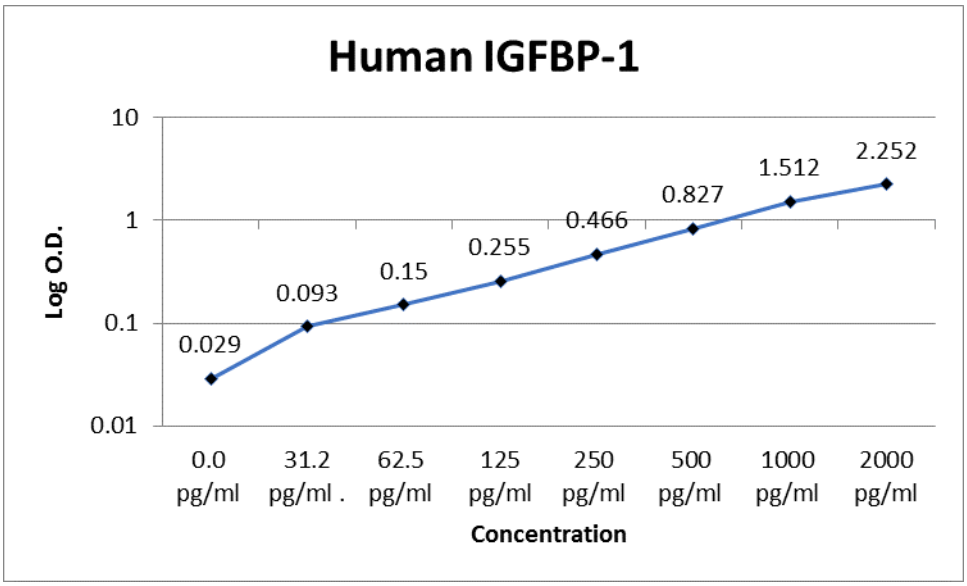
(b) PC Interface Plate Reading

Enter the data into computer program curve fitting software. Existing spreadsheet software can perform a good fit with a linear regression analysis.

IX: Typical Standard Curve (for reference only, not to be used for actual data)

Concentration pg/ml	0.0 pg/ml	31.2 pg/ml .	62.5 pg/ml	125 pg/ml	250 pg/ml	500 pg/ml	1000 pg/ml	2000 pg/ml
O.D.	0.029	0.093	0.150	0.255	0.466	0.827	1.512	2.252





TMB reaction incubated at 37°C for 15-20 minutes

X: Appendix A Acidification/Neutralization Procedure for pretreatment of protein and blood samples

Needed solutions:
Solution A: 1N HCI, pH < 1.0: add 8.33ml of 12N HCI into 91.67ml of H2O.
Solution B: 1.2N NaOH/0.5M HEPES, pH 14: add 12ml of 10N NaOH and 11.9g HEPES into 75ml of H2O, add H2O to adjust volume to 100ml.

Cell culture supernatant, urine: (1:5 treatment ratio): Add acidification reagent proportionally, i.e. add 20 microliters of Solution A into 100 microliters of undiluted sample, 10 min later, add 20 microliters of Solution B to the tube to neutralize the acidification step. If precipitate forms, samples can be centrifuged at 16,000 x g for 5 minutes at 4 degrees centigrade to pellet any material. Use only the clear supernatants for the ELISA. The final pH of the sample solution should be ~7.0 to 7.6 for use in the ELISA. Use pH paper or microprobe to check final pH.

Serum, plasma (EDTA,heparin): (1:2 treatment ratio) Add acidification reagent proportionally, i.e. add 20 microliters of Solution A into 40 microliters of undiluted sample, 10 min later, add 20 microliters of Solution B to the tube to neutralize the acidification step. If precipitate forms, samples can be centrifuged at 16,000 x g for 5 minutes at 4 degrees centigrade to pellet any material. Use only the clear supernatants for the ELISA. The final pH of the sample solution should be ~7.0 to 7.6 for use in the ELISA. Use pH paper or microprobe to check final pH.

Note: Sample acidification/neutralization will dilute experimental samples partly after adding the reagents, so please pay attention to this when calculating target protein concentration for the most accurate results.

Also, the user needs to estimate the concentration of the target protein in the sample and select a proper dilution factor so that the diluted target protein concentration falls near the middle of the linear regime in the standard curve. Moreover with certain growth factors and target proteins, binding proteins and location can influence over all signal. It is recommended that researchers confirm by literature an effective lysis, cell membrane preparations and/or extraction buffer formulations and treatments that are suitable for the precise target being assayed.