CHICKEN INSULIN LIKE GROWTH FACTOR BINDING PROTEIN 3 ELISA KIT INSTRUCTION MANUAL

This product is for research use **ONLY** and not for

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Catalogue No.: LZ00075
Size: 48T/96T
Reactivity: Chicken
Range: 6.25-400ng/ml
Sensitivity: <3.75ng/ml
Application: For quantitative detection of Insulin Like Growth Factor Binding Protein 3 (IGFBP-3) in serum, plasma, tissue homogenates and other biological fluids.
Storage: 4°C for 6 months

Contents

I. Introduction

Toronto Research Chemicals, TRC was founded in 1982 to manufacture and supply researchers in the biomedical fields with specialized complex organic small molecules not otherwise commercially available. In order to provide new research tools to life scientists, we now offer antibodies, ELISA kits, screening libraries, DNA ladders and certified reference materials.

ELISA is one of the most sensitive and reproducible technologies available. These assays are rapid, simple to perform and easily automated.

As with any assay, the reproducibility and reliability of ELISAs depend upon proper techniques and attention to detail. This ELISA instruction manual will increase your awareness of ELISA techniques and help you maintain proficiency with this methodology.

Check your package inserts for specific instructions for each assay you perform. Periodically improvements and revisions are made to package inserts to support your research in a more efficient manner. If you have questions concerning any of the following information, call TRC Technical Assistance, contact information listed below.

II. Technical Assistance

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III. Abbreviations

I. **TMB:** 3,3',5,5'-Tetramethylbenzidine
II. **EDTA:** Ethylenediaminetetraacetic Acid
III. **PBS:** Phosphate buffered saline
IV. **SABC:** HRP-Streptavidin

IV. Supplied Materials

Item	Specifications (48T/96T)	Storage
Micro ELISA Plate (Dismountable)	8×6/8×12	4°C/-20°C
Lyophilized Standard	1 vial/2 vial	4°C/-20°C
Sample / Standard dilution buffer	10 ml/20 ml	4°C
Biotin-detectionAntibody (Concentrated)	60 µl /120 µl	4°C
Antibody dilution buffer	5 ml/10 ml	4°C
HRP-Streptavidin Conjugate (SABC)	60 µl /120 µl	4°C
SABC dilution buffer	5 ml/10 ml	4°C
TMB substrate	5 ml/10 ml	4°C
Stop solution	5 ml/10 ml	4°C
Wash buffer (25X)	15 ml/30 ml	4°C
Plate Sealer	3/5 pieces	
Product Description	1 copy	

Please Note:

- Do not use the kit after the expiration date.
- Sample diluent is designed for dilution of standard and samples. Please use appropriate sample diluents based on the sample type.
- Detection diluent is designed for dilution of detection

V. Additional Required Materials

1. A microplate reader capable of measuring absorbance at 450 nm with the correction wavelength set at 630 nm.
2. Calibrated, adjustable precision pipettes and disposable plastic tips.
   (A manifold multi-channel pipette is recommended for large assays.)
3. Distilled, deionized water.
4. 37°C incubator
5. Plate washer: Automated or Manual.
6. Glass or plastic tubes to prepare standard and sample dilutions.
7. Absorbent paper towels.
8. Beakers and graduated cylinders.
9. Log-log or semi-log graph paper or graphing software for ELISA data analysis.

VI. Principle Of Assay

This kit is based on sandwich enzyme-linked immune-sorbent assay (ELISA) technology. Anti-IGFBP-3 antibodies are pre-coated onto 96-well mircoplate, with biotin conjugated anti-IGFBP-3 antibodies used for detection. The standards, test samples and biotin conjugated detection antibodies are all added to the wells subsequently, and wash with wash buffer. HRP-Streptavidin is then added, with unbound conjugates being washed away with wash buffer. TMB substrates are then used to visualize the HRP enzymatic reaction. TMB is catalyzed by HRP to produce a blue colored product that changes to yellow after the addition of acidic stop solution. The density of this yellow product is proportional to the IGFBP-3 amount of sample captured within each well. Lastly, the concentration of IGFBP-3 within each sample can be calculated, by comparing its absorbance at 450nm to that of the standard curve prepared.

I. Precautions for Use

1. To inspect the validity of experimental operation, as well as, the appropriateness of sample dilution proportion, a pilot experiment using standards and a small number of samples is recommended.
2. All samples and reagents must be at room temperature (20-25°C) before use in the assay.
3. After opening and before using, keep plate dry.
4. Before using the Kit, briefly centrifuge the tubes of reagents before use.
5. Avoid exposing reagents to excessive heat or light during storage and incubation.
6. The washing process is very important, improper washing can easily result in false positives.
7. Duplicate well assay is recommended for both standard and sample testing.
8. Don't let microplate dry during the assay, as a dry plate will inactivate components on plate.
9. Don't reuse tips and tubes as to avoid cross contamination.
10. Avoid using the reagents from different batches together.

VII. Assay Preparation

2. Sample Preparation

Isolate the test samples soon after collecting, then, analyze immediately (within 2 hours) or aliquot and store at -20°C. Avoid multiple freeze-thaw cycles.

A. Serum: Allow blood samples to clot for 2 hours at room temperature or overnight at 4°C prior to centrifugation for 20 minutes at approximately 1000×g. Collect the supernatant and carry out the assay immediately. Blood collection tubes should be disposable, non-pyrogenic, and non-endotoxin.

B. Plasma: Collect plasma using EDTA-Na_2 as an anticoagulant. Centrifuge samples for 15 minutes at 1000×g at 2-8°C within 30 minutes of collection. Collect the supernatant and carry out the assay immediately. The plasma can be assayed immediately or aliqouted and stored at -20°C. Avoid multiple freeze-thaw cycles.

Please Note:

- Do not use turbid or grossly hemolyzed samples. Mix thawed samples thoroughly before starting the ELISA assay.

C. Tissue homogenates: For general information, hemolysis blood may affect the result, so you should rinse the tissues with ice-cold PBS (0.01M, pH=7.4) to remove excess blood thoroughly. Tissue pieces should be weighed and then minced to small pieces which will be homogenized in PBS (the volume depends on the weight of the tissue. 9mL PBS would be appropriate to 1 gram of tissue. Some protease inhibitor is recommended to add into the PBS.) with a glass homogenizer on ice. To further break the cells, you can sonicate the suspension with an ultrasonic cell disrupter or subject it to freeze-thaw cycles. The homogenates are then centrifuged for 5 minutes at 5000×g to acquire the supernatant.

D. Cell culture supernatant: Centrifuge supernatant for 20 minutes to remove insoluble impurity and cell debris at 1000×g at 2-8°C. Collect the clear supernatant and carry out the assay immediately or aliquot and store at -20°C. Avoid multiple freeze-thaw cycles.

2. Sample Preparation

E. Other biological fluids: Centrifuge samples for 20 minutes at 1000×g at 2 - 8°C. Collect the supernatant and carry out the assay immediately.

Please Note:

- Samples should be clear and transparent as well as be centrifuged to remove suspended solids.

Samples should be used within 5 days stored at 4°C, otherwise samples must be stored at -20°C (≤1 month) or -80°C (≤2 months) to avoid loss of bioactivity and contamination. Hemolyzed samples are not suitable for use in this assay

3. Reagent Preparation

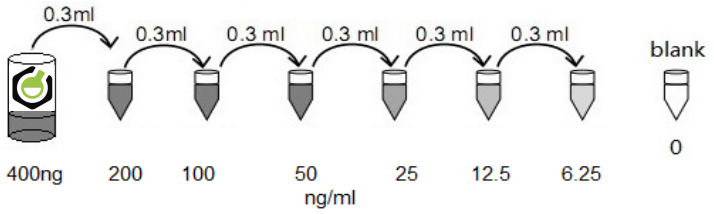
Bring all reagents to room temperature before use.

1. Wash Buffer:

Dilute 30mL of Concentrated Wash Buffer into 750 mL of Wash Buffer with deionized or distilled water, store unused solution at 4°C. If crystals have formed in the concentrate, you can warm it with 40°C water bath (Ensure heating temperature should not exceed 50°C) and mix it gently until the crystals have completely dissolved. The solution should be cooled to room temperature before use.

2. Standards:

1. **400ng/ml standard solution**: Add 1 ml of the Sample / Standard dilutions buffer to Lyophilized Standard tube, keep the tube at room temperature for 10 min and mix thoroughly.
2. **200ng/ml-6.25ng/ml standard solutions**: Label 6 tubes with 200ng/ml, 100ng/ml, 50ng/ml, 25ng/ml, 12.5ng/ml, 6.25ng/ml, respectively. Aliquot 0.3 ml of the Sample / Standard dilution buffer into each tube. Add 0.3 ml of the above 400ng/ml standard solution into 1st tube and mix thoroughly. Transfer 0.3 ml from 1st tube to 2nd tube and mix thoroughly. Transfer 0.3 ml from 2nd tube to 3rd tube and mix thoroughly, and so on.



3. Reagent Preparation

Please Note:

- The standard solutions are best used within 2 hours of preparation. The standard solution should be at 4°C for up to 12 hours or stored at -20 °C for up to 48 hours. Avoid repeated freeze-thaw cycles.

3. Preparation of Biotin-detection Antibody working solution:

Prepare within 1 hour before the experiment.

1. Calculate the total volume of the working solution: 0.1 ml / well × quantity of wells. (Allow 0.1-0.2 ml more than the total volume)
2. Dilute the Biotin-detection antibody with Antibody dilution buffer at 1:100 and mix thoroughly.(i.e. Add 1 µl of Biotin-detection antibody into 99 µl of Antibody dilution buffer.)

4. Preparation of HRP-Streptavidin Conjugate (SABC) working solution:

Prepare within 30 minutes before the experiment.

1. Calculate the total volume of the working solution: 0.1 ml / well × quantity of wells. (Allow 0.1-0.2 ml more than the total volume)
2. Dilute the SABC with SABC dilution buffer at 1:100 and mix thoroughly. (i.e. Add 1 µl of SABC into 99 µl of

VIII. Assay Procedure

Before adding to wells, equilibrate the SABC working solution and TMB substrate for at least 30 minutes at 37 °C. When diluting samples and reagents, they must be mixed completely and evenly. It is recommended to plot a standard curve for each test.

1. Set standard, test sample and control wells on the pre-coated plate respectively, and then, record their positions. It is recommended to measure each standard and sample in duplicates. **Wash plate 2 times before adding to the standard, sample and control wells!**
2. Aliquot 0.1ml of 400ng/ml, 200ng/ml, 100ng/ml, 50ng/ml, 25ng/ml, 12.5ng/ml, 6.25ng/ml, standard solutions into the standard wells.
3. Add 0.1 ml of Sample / Standard dilution buffer into the control (zero) well.
4. Add 0.1 ml of properly diluted sample (serum, plasma, tissue homogenates and other biological fluids.) into test sample wells.

VIII. Assay Procedure

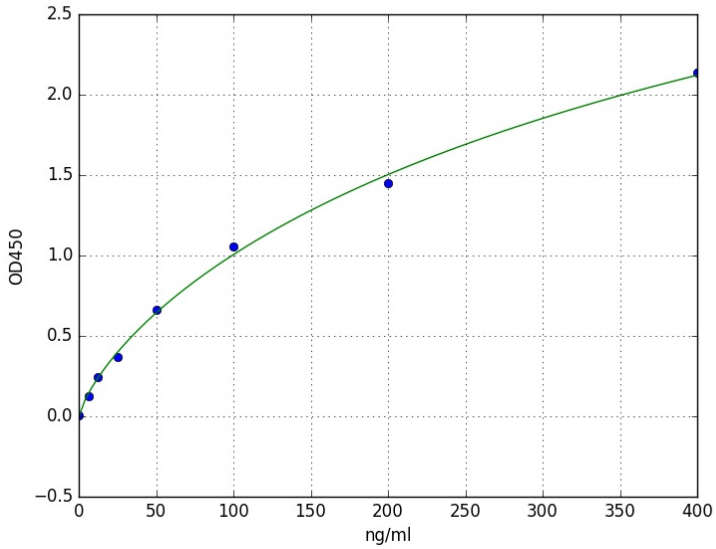
5. Seal the plate with a cover and incubate at 37 °C for 90 min.
6. Remove the cover and discard the plate content, firmly tap the plate on the absorbent filter papers or other absorbent material. Do NOT let the wells completely dry at any time. **Do Not Wash Plate!**
7. Add 0.1 ml of Biotin-detection antibody working solution into each well (standard, test sample & zero wells). Add the solution to the bottom of each well without touching the sidewalls.
8. Seal the plate with a cover and incubate at 37°C for 60 min.
9. Remove the cover, and wash plate 3 times with Wash buffer.
10. Add 0.1 ml of SABC working solution into each well, cover the plate and incubate at 37°C for 30 min.
11. Remove the cover and wash plate 5 times with Wash buffer, and each time let the wash buffer stay in the wells for 1-2 min.
12. Add 90 µl of TMB substrate into each well, cover the plate and incubate at 37°C in dark within 15-30 min. (Note: This incubation time is for reference use only, the optimal time should be determined by end user.) And the shades of blue can be seen in the first 3-4 wells (with most concentrated IGFBP-3 standard solutions), the other wells show no obvious color.
13. Add 50 µl of Stop solution into each well and mix thoroughly. The color changes into yellow immediately.
14. Read the O.D. absorbance at 450 nm in a microplate reader immediately after adding the stop solution.

For calculation, (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). The IGFBP-3 concentration of the samples can be interpolated from the standard curve. Recommended to use professional software curve expert to 1.3.

Please Note:

- If the samples measured were diluted, multiply the dilution factor to the concentrations from interpolation to obtain the concentration before dilution.

IX. Data Analysis



4. Standard Curve

Results of a typical standard run of an IGFBP-3 ELISA Kit are shown above. This standard curve was generated at our lab for demonstration purpose only. Each user should obtain their own standard curve as per experiment.

5. Specificity

This assay has high sensitivity and excellent specificity for the detection of IGFBP-3. No significant cross-reactivity or interference between IGFBP-3 and analogues was observed.

Please Note:

- Limited by current skills and knowledge, it is impossible for us to complete the cross- reactivity detection between IGFBP-3 and all the analogues, therefore, cross-reaction may still exist.

6. Recovery

Matrices listed below were spiked with certain level of IGFBP-3 and the recovery rates were calculated by comparing the measured value to the expected amount of IGFBP-3 in samples.

Matrix	Recovery range (%)	Average (%)
Serum (n=5)	85-101	82
EDTA plasma (n=5)	85-100	93
Heparin plasma (n=5)	88-105	94

7. Linearity

The linearity of the kit was assayed by testing samples spiked with appropriate concentration of IGFBP-3 and their serial dilutions. The results were demonstrated by the percentage of calculated concentration to the expected.

Sample	1:2	1:4	1:8	1:16
Serum (n=5)	91-101%	86-99%	86-105%	88-104%
EDTA plasma (n=5)	85-100%	84-98%	83-95%	85-98%
Heparin plasma (n=5)	88-105%	84-97%	85-91%	80-94%

8. Precision

Intra-assay Precision (Precision within an assay): 3 samples with low, middle and high levels of IGFBP-3 were tested 20 times on one plate, respectively.

Inter-assay Precision (Precision between assays): 3 samples with low, middle and high levels of IGFBP-3 were tested on 3 different plates, 8 replicates in each plate.

CV (%) = SD/meanX100

Intra-Assay: CV<8%

Inter-Assay: CV<10%

9. Stability

The stability of ELISA kit is determined by the loss rate of activity. The loss rate of this kit is less than 10% within the expiration date under appropriate storage condition.

Standard (n=5)	37°C for 1 months	4°C for 6 months
Average (%)	80	95-100

To minimize extra influence on the performance, operation procedures and lab conditions, especially room temperature, air humidity, incubator temperature should be strictly controlled. It is also strongly suggested that the whole assay be performed by the same operator from beginning to end.

XI. Additional Information

10. Washing Procedure

1. Manual Washing

Discard the solution in the plate without touching the sidewalls. Firmly tap the plate on absorbent filter paper or other absorbent material. Fill each well completely with 350 µl of wash buffer and soak for 1 to 2 minutes. Then aspirate contents from the plate, and clap the plate on absorbent filter paper or other absorbent material. Repeat this procedure two more times for a total of THREE washes.

2. Automated Washing

Aspirate all wells, and then wash plate THREE times with 350 µl of wash buffer. After the final wash, invert plate, and firmly tap the plate on absorbent filter paper or other absorbent material. It is recommended that the washer be set for a soaking time of 1 minute.

11. Sample Dilution Guidelines

End user should estimate the concentration of the target protein in the test sample first, and select a proper dilution factor to make the diluted target protein concentration falls the optimal detection range of the kit. Dilute the sample with the provided dilution buffer; several trials may be necessary in practice. The test sample must be well mixed with the dilution buffer.

I. **High target protein concentration (4000-40000ng/ml):** Dilution: 1:100. (I.e. Add 1µl of sample into 99 µl of Sample/Standard dilution buffer.)

II. **Medium target protein concentration (400-4000ng/ml):** Dilution: 1:10. (I.e. Add 10 µl of sample into 90 µl of Sample/Standard dilution buffer.)

III. **Low target protein concentration (6.25-400ng/ml):** Dilution: 1:2. (I.e. Add 50 µl of sample into 50 µl of Sample/Standard dilution buffer.)

IV. **Very low target protein concentration (≤6.25ng/ml):** Unnecessary to dilute, or dilute at 1:2.

Visit our website at www.trc-canada.com for MSDS, troubleshooting tips and other related literature for our ELISA kits.

XII. Resources