KAMIYA BIOMEDICAL COMPANY

Human IL-6 ELISA

**For the quantitative determination of IL-6
in human cell culture supernates, serum and plasma (heparin, EDTA, citrate)**

Cat. No. KT-1348

For Research Use Only. Not for diagnostic use in the U.S.

PRODUCT INFORMATION

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INTENDED USE
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INTRODUCTION
The human interferon-beta 2 gene (IFNB2) product is identical to that for the B-cell stimulation factor-2 (BSF-2), the hybridoma growth factor (HGF) ("interleukin-6"), and the hepatocyte stimulating factor (HSF). Proteins derived from this gene mediate the plasma protein response to tissue injury (acute-phase response) and regulate the growth and differentiation of both B and T cells. Interleukin-6 (IL6) has come to be regarded as a potential osteoporotic factor because it has stimulatory effects on cells of the osteoclast lineage, and, thus, may play a role in the pathogenesis of bone loss associated with estrogen deficiency. IL-6 has many roles essential to the regulation of the immune response, hematopoiesis, and bone resorption. It is involved not only in the hepatic acute phase response but also in adipose tissue metabolism, lipoprotein lipase activity, and hepatic triglyceride secretion. Overproduction of IL-6, a proinflammatory cytokine, is associated with a spectrum of age-related conditions including cardiovascular disease, osteoporosis, arthritis, type 2 diabetes, certain cancers, periodontal disease, frailty, and functional decline. BSF-2 is a novel interleukin consisting of 184 amino acids. The calibrator product used in this kit is recombinant human IL-6, consisting of 184 amino acids with the molecular mass of 20.3 kDa.

PRINCIPLE
Human IL-6 ELISA Kit was based on standard sandwich enzyme-linked immune-sorbent assay technology. A antibody specific for IL-6 has been precoated onto 96-well plates. Calibrators (Expression system for calibrator: E.coli; Immunogen sequence: P29-M212) and test samples are added to the wells, a biotinylated detection antibody specific for IL-6 is added subsequently and then followed by washing with PBS or TBS buffer. Avidin-Biotin-Peroxidase Complex was added and unbound conjugates were washed away with PBS or TBS buffer. HRP substrate TMB was used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic stop solution. The density of yellow is proportional to the Human IL-6 amount of sample captured in plate.

COMPONENTS
1. 96-well plate precoated with anti-human IL-6 antibody: 1

2. lyophilized recombinant human IL-6 calibrator: 10 ng/tube x 2

3. biotinylated anti-human IL-6 antibody: 130 µL (dilution 1:100)

4. Avidin-Biotin-Peroxidase Complex (ABC): 130 µL (dilution 1:100)

5. sample diluent buffer: 30 mL

6. antibody diluent buffer: 12 mL

7. ABC diluent buffer: 12 mL

8. TMB color developing agent: 10 mL

9. TMB stop solution: 10 mL

10. Adhesive cover: 4

MATERIALS REQUIRED BUT NOT PROVIDED
1. Microplate reader in standard size.
2. Automated plate washer.

3. Adjustable pipettes and pipette tips. Multichannel pipettes are recommended in the condition of large amount of samples in the detection.
4. Clean tubes and Eppendorf tubes.
5. Washing buffer (neutral PBS or TBS).
-Preparation of 0.01M **TBS:** Add 1.2 g Tris, 8.5 g NaCl; 450 µL of purified acetic acid or 700µL of concentrated hydrochloric acid to 1,000 mL distilled water and adjust pH to 7.2~7.6. Finally, adjust the total volume to 1 L.
-Preparation of 0.01 M **PBS:** Add 8.5 g sodium chloride, 1.4 g Na_2HPO_4 and 0.2 g NaH_2PO_4 to 1,000 mL distilled water and adjust pH to 7.2~7.6. Finally, adjust the total volume to 1 L.

PRECAUTIONS

Please read the following instructions before starting the experiment.
1. To inspect the validity of experiment operation and the appropriateness of sample dilution proportion, pilot experiment using calibrators and a small number of samples is recommended.
2. The TMB Color Developing agent is colorless and transparent before using, contact us freely if it is not the case.
3. Before using the Kit, spin tubes and bring down all components to the bottom of tubes.
4. Duplicate well assay is recommended for both calibrator and sample testing.
5. Don't let 96-well plate dry, for dry plate will inactivate active components on plate.
6. Don't reuse tips and tubes to avoid cross contamination.
7. Avoid using the reagents from different batches together.
8. In order to avoid marginal effect of plate incubation due to temperature difference (reaction may be stronger in the marginal wells), it is suggested that the diluted ABC and TMB solution will be pre-warmed in 37℃ for 30 min before using.
9. Take precautionary measures to prevent operator contamination (such as saliva and other body fluids) of kit reagents while running this assay.

REAGENT PREPARATION

1. **Sample Preparation and Storage**
Store samples to be assayed within 24 hours at 4℃. For long-term storage, aliquot and freeze samples at -20℃. Avoid repeated freeze-thaw cycles.
-Serum: Allow the serum to clot in a serum separator tube (about 4 hours) at room temperature. Centrifuge at approximately 1,000 X g for 15 min. Analyze the serum immediately or aliquot and store samples at -20℃.
-Cell culture supernates: Remove particulates by centrifugation, assay immediately or aliquot and store samples at -20℃.
-Plasma: Collect plasma using heparin, EDTA or citrate as an anticoagulant. Centrifuge for 15 min at 1,500 x g within 30 min of collection. Assay immediately or aliquot and store samples at -20℃.

2. **Sample Dilution Guideline**
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 calibration curve. Dilute the sample using the provided diluent buffer. The following is a guideline for sample dilution. Several trials may be necessary in practice. **The sample must be well mixed with the diluents buffer.**

-High target protein concentration (3,000 pg/mL - 30,000 pg/mL). The working dilution is 1:100. i.e. Add 1 µL sample into 99 µL sample diluent buffer.
-Medium target protein concentration (300 pg/mL - 3,000 pg/mL). The working dilution is 1:10. i.e. Add 10 µL sample into 90 µL sample diluent buffer.
-Low target protein concentration (4.69 pg/mL - 300 pg/mL). The working dilution is 1:2. i.e. Add 50 µL sample to 50 µL sample diluent buffer.
-Very Low target protein concentration (0 pg/mL - 4.69 pg/mL). No dilution necessary, or the working dilution is 1:2.

3. **Reagent Preparation and Storage**
A. Reconstitution of the Human IL-6 calibrator: IL-6 calibrator solution should be prepared no more than 2 hours prior to the experiment. Two tubes of IL-6 calibrator (10 ng per tube) are included in each kit. Use one tube for each experiment.
a. 10,000 pg/mL of Human IL-6 calibrator solution: Add 1 mL sample diluent buffer into one tube, keep the tube at room temperature for 10 min and mix thoroughly.
b. 300 pg/mL of Human IL-6 calibrator solution: Add 0.03 mL of the above IL-6 calibrator solution into 0.97 mL sample diluent buffer and mix thoroughly.
c. 150 pg/mL→4.6875 pg/mL of Human IL-6 calibrator solutions: Label 6 Eppendorf tubes with 150 pg/mL, 75 pg/mL, 37.5 pg/mL, 18.75 pg/mL, 9.375 pg/mL, 4.6875 pg/mL respectively. Aliquot 0.3 mL of the sample diluent buffer into each tube. Add 0.3 mL of the above 300 pg/mL IL-6 calibrator solution into 1st tube and mix. Transfer 0.3 mL from 1st tube to 2nd tube and mix. Transfer 0.3 mL from 2nd tube to 3rd tube and mix, and so on.

Note: The calibrator solutions are best used within 2 hours. The 10,000 pg/mL calibrator solution should be stored at 4℃ for up to 12 hours, or at -20℃ for up to 48 hours. Avoid repeated freeze-thaw cycles.
B. Preparation of biotinylated anti-Human IL-6 antibody working solution: The solution should be prepared no more than 2 hours prior to the experiment.
a. The total volume should be: 0.1 mL/well x (the number of wells). (Allowing 0.1-0.2 mL more than total volume)
b. Biotinylated anti-Human IL-6 antibody should be diluted in 1:100 with the antibody diluent buffer and mixed thoroughly. (i.e. Add 1 µL Biotinylated anti-Human IL-6 antibody to 99 µL antibody diluent buffer.)
C. Preparation of Avidin-Biotin-Peroxidase Complex (ABC) working solution: The solution should be prepared no more than 1 hour prior to the experiment.
a. The total volume should be: 0.1 mL/well x (the number of wells). (Allowing 0.1-0.2 mL more than total volume)
b. Avidin- Biotin-Peroxidase Complex (ABC) should be diluted in 1:100 with the ABC dilution buffer and mixed thoroughly. (i.e. Add 1 µL ABC to 99 µL ABC diluent buffer.)

STORAGE

Store at 4˚C for 6 months, at -20˚C for 12 months. Avoid multiple freeze-thaw cycles.

ASSAY PROTOCOL

The ABC working solution and TMB color developing agent must be kept warm at 37℃ for 30 min before use. When diluting samples and reagents, they must be mixed completely and evenly. Calibrator IL-6 detection curve should be prepared for each experiment. The user will decide sample dilution fold by crude estimation of IL-6 amount in samples.

1. Aliquot 0.1 mL per well of the 300 pg/mL, 150 pg/mL, 75 pg/mL, 37.5 pg/mL, 18.75 pg/mL, 9.375 pg/mL, 4.6875 pg/mL Human IL-6 calibrator solutions into the precoated 96-well plate. Add 0.1 mL of the sample diluent buffer into the control well (Zero well). Add 0.1 mL of each properly diluted sample of Human cell culture supernates, serum or plasma (heparin, EDTA, citrate) to each empty well. **See "Sample Dilution Guideline" above for details.** It is recommended that each Human IL-6 calibrator solution and each sample be measured in duplicate.
2. Seal the plate with a new adhesive cover provided and incubate at 37℃ for 90 min.
3. Remove the cover, discard plate content, and blot the plate onto paper towels or other absorbent material. Do NOT let the wells completely dry at any time.
4. Add 0.1 mL of biotinylated anti-Human IL-6 antibody working solution into each well, seal the plate with a new adhesive cover provided and incubate at 37℃ for 60 min.
5. Wash plate 3 times with 0.01M TBS or 0.01M PBS, and each time let washing buffer stay in the wells for 1 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material. (**Plate Washing Method:** Discard the solution in the plate without touching the side walls. Blot the plate onto paper towels or other absorbent material. Soak each well with at least 0.3 mL PBS or TBS buffer for 1~2 minutes. Repeat this process two additional times for a total of THREE washes. Note: For automated washing, aspirate all wells and wash THREE times with PBS or TBS buffer, overfilling wells with PBS or TBS buffer. Blot the plate onto paper towels or other absorbent material.)
6. Add 0.1 mL of prepared ABC working solution into each well, seal the plate with a new adhesive cover provided and incubate at 37℃ for 30 min.
7. Wash plate 5 times with 0.01M TBS or 0.01M PBS, and each time let washing buffer stay in the wells for 1-2 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material. (See Step 5 for plate washing method.)
8. Add 90 µL of prepared TMB color developing agent into each well, seal the plate with a new adhesive cover and incubate at 37℃ in dark for 20-25 min (**Note:** For reference only, the optimal incubation time should be determined by end user. And the shades of blue can be seen in the wells with the four most concentrated Human IL-6 calibrator solutions; the other wells show no obvious color).
9. Add 0.1 mL of prepared TMB stop solution into each well. The color changes into yellow immediately.
10. Read the O.D. absorbance at 450 nm in a microplate reader within 30 min 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 calibration curve can be plotted as the relative O.D.450 of each calibrator solution (Y) vs. the respective concentration of the calibrator solution (X). The Human IL-6 concentration of the samples can be interpolated from the calibration curve.
Note: if the samples measured were diluted, multiply the dilution factor to the concentrations from interpolation to obtain the concentration before dilution.

SUMMARY

1. Add samples and calibrators and incubate the plate at 37℃ for 90 min. Do not wash.
2. Add biotinylated antibodies and incubate the plate at 37℃ for 60 min. Wash plate 3 times with 0.01M TBS.
3. Add ABC working solution and incubate the plate at 37℃ for 30 min. Wash plate 5 times with 0.01M TBS.
4. Add TMB color developing agent and incubate the plate at 37℃ in dark for 20-25 min.

5. Add TMB stop solution and read.

PERFORMANCE CHARACTERISTICS

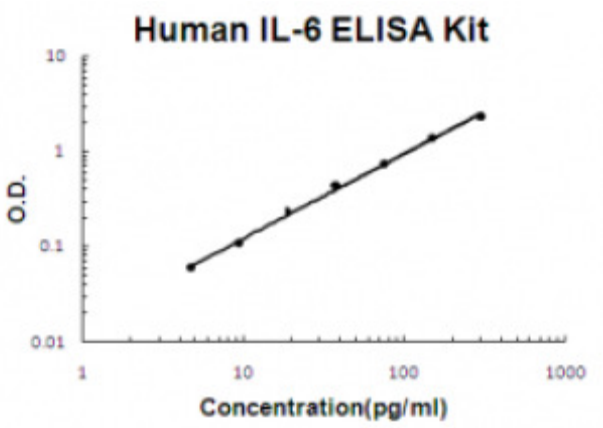
Typical Data Obtained from Human IL-6

(TMB reaction incubate at 37°C for 20-25min)

Concentration(pg/ml)	0	4.69	9.38	18.75	37.5	75	150	300
O.D.	0.002	0.059	0.106	0.226	0.428	0.736	1.372	2.279

Typical Human IL-6 ELISA Kit Calibration Curve

This calibration curve was generated for demonstration purpose only. A calibration curve must be run with each assay.



Range: 4.69 pg/mL - 300 pg/mL
Sensitivity: <0.3 pg/mL
Specificity: Natural and recombinant Human IL-6
Cross-reactivity: There is no detectable cross-reactivity with other relevant proteins.

Intra-Assay Precision (Precision within an assay) Three samples of known concentration were tested on one plate to assess intra-assay precision.
Inter-Assay Precision (Precision between assays) Three samples of known concentration were tested in separate assays to assess inter-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)	16.3	98	179	18.2	99	185
Standard deviation	0.8	2.3	4.2	1.0	3.6	5.7
CV(%)	4.9	2.3	2.3	5.5	3.6	3.1

FOR RESEARCH USE ONLY

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