KAMIYA BIOMEDICAL COMPANY

Human TNFsR I ELISA

For the quantitative determination of TNFsR I in human cell culture supernates, serum, plasma (heparin, EDTA, citrate) and urine

Cat. No. KT-1505

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

PRODUCT INFORMATION

Human TNFsR I ELISA
Cat. No. KT-1505

INTENDED USE
The Human TNFsR I ELISA is for the quantitative determination of TNFsR I in human cell culture supernates, serum, plasma (heparin, EDTA, citrate) and urine. For research use only. Not for diagnostic use in the U.S.

INTRODUCTION
TNFsR I (also known as Solube tumor necrosis factor receptor 1) is a cytokine receptor that binds tumor necrosis factors (TNF). In contrast, the p60 (TNFRSF1A) and p80 (TNFRSF1B) TNFA receptors self-assemble through a distinct functional domain in the TNFR extracellular domain, termed the pre-ligand assembly domain (PLAD), in the absence of ligand. Deletion of the PLAD results in monomeric presentation of p60 or p80. Flow cytometric analysis showed that efficient TNFA binding depends on receptor self-assembly. They also found that other members of the TNF receptor superfamily, including the extracellular domains of TRAIL (TNFRSF10A), CD40, and FAS (TNFRSF6), all self-associate but do not interact with heterologous receptors. By Southern blot analysis of human/Chinese hamster somatic cell hybrid DNA, the TNFR1 gene was mapped to 12pter-cen. And by nonradioactive in situ hybridization that the type 1 receptor (the p55 TNF receptor) is encoded by a gene located on chromosome 12p13.2.

PRINCIPLE
The human TNFsR I ELISA Kit was based on standard sandwich enzyme-linked immune-sorbent assay technology. A monoclonal antibody from mouse specific for TNFsR I has been precoated onto 96-well plates. Calibrators (NSO, L30-T211) and test samples are added to the wells, a biotinylated detection polyclonal antibody from goat specific for TNFsR I 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 TNFsR I amount of sample captured in plate.

COMPONENTS
1. 96-well plate precoated with anti-human TNFsR I antibody: 1

2. lyophilized recombinant human TNFsR I calibrator: 10 ng/tube x 2

3. biotinylated anti-human TNFsR I 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

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 0.01 M **TBS:** Add 1.2 g Tris, 8.5 g Nacl; 450 µL of purified acetic acid or 700 µL of concentrated hydrochloric acid to 1, 000 mL H_2O 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

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.

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.

-**Cell culture supernates**: Remove particulates by centrifugation, assay immediately or aliquot and store samples at -20℃.

-**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℃.

-**Plasma**: Collect plasma using **heparin, EDTA or citrate** as an anticoagulant. Centrifuge for 15 min at 1,000 x g within 30 min of collection. Assay immediately or aliquot and store samples at -20℃.

-**Urine**: Aseptically collect the first urine of the day, micturate directly into a sterile container. Remove particular impurities by centrifugation, 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 (5-50 ng/mL). The working dilution is 1:100. i.e. Add 1 µL sample into 99 µL sample diluent buffer.
-Medium target protein concentration (500-5,000 pg/mL). The working dilution is 1:10. i.e. Add 10 µL sample into 90 µL sample diluent buffer.
-Low target protein concentration (7.8-500 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 (≤7.8 pg/mL).** No dilution necessary, or the working dilution is 1:2.

3. **Reagent Preparation and Storage**

A. Reconstitution of the human TNFsR I calibrator: TNFsR I calibrator solution should be prepared no more than 2 hours prior to the experiment. Two tubes of TNFsR I calibrator (10 ng per tube) are included in each kit. Use one tube for each experiment.
a. 10,000 pg/mL of human TNFsR I calibrator solution: Add 1 mL sample diluent buffer into one tube, keep the tube at room temperature for 10 min and mix thoroughly.
b. 500 pg/mL of human TNFsR I calibrator solution: Add 0.05 mL of the above 10 ng/mL TNFsR I calibrator solution into 0.95 mL sample diluent buffer and mix thoroughly.
c. 250 pg/mL→7.8 pg/mL of human TNFsR I calibrator solutions: Label 6 Eppendorf tubes with 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, 15.6 pg/mL, 7.8 pg/mL respectively. Aliquot 0.3 mL of the sample diluent buffer into each tube. Add 0.3 mL of the above 500 pg/mL TNFsR I 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 ng/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 TNFsR I 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 TNFsR I antibody should be diluted in 1:100 with the antibody diluent buffer and mixed thoroughly. (i.e. Add 1 µL Biotinylated anti-human TNFsR I 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 TNFsR I detection curve should be prepared for each experiment. The user will decide sample dilution fold by crude estimation of TNFsR I amount in samples.
1. Aliquot 0.1 mL per well of the 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, 15.6 pg/mL, 7.8 pg/mL human TNFsR I 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) to each empty well. **See "Sample Dilution Guideline" above for details.** It is recommended that each human TNFsR I calibrator solution and each sample be measured in duplicate.
2. Seal the plate with the cover 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 TNFsR I antibody working solution into each well and incubate the plate at 37℃ for 60 min.
5. Wash plate 3 times with 0.01 M TBS or 0.01 M 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 and incubate the plate at 37℃ for 30 min.
7. Wash plate 5 times with 0.01 M TBS or 0.01 M 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 and incubate plate at 37℃ in dark for 15-20 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 TNFsR I 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 TNFsR I 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 15-20 min.
5. Add TMB stop solution and read.

PERFORMANCE CHARACTERISTICS

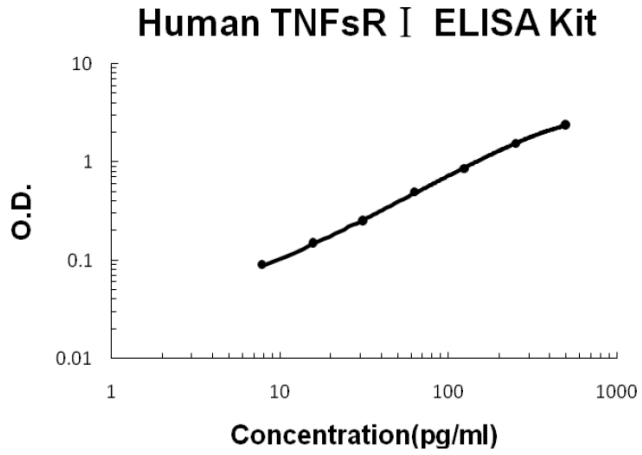
Typical Data Obtained from Human TNFsR I

(TMB reaction incubate at 37°C for 15 min)

Concentration(pg/ml)	0.0	7.8	15.6	31.3	62.5	125	250	500
O.D.	0.024	0.092	0.149	0.249	0.499	0.871	1.574	2.361

Typical Human TNFsR I ELISA Kit Calibration Curve

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



Range: 7.8 pg/mL - 500 pg/mL
Sensitivity: <1 pg/mL
Specificity: Natural and Recombinant Human TNFsR I
Cross-reactivity: 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)	74	133	261	85	156	278
Standard deviation	2.52	6.52	14.9	3.91	7.96	18.07
CV(%)	3.4	4.9	5.7	4.6	5.1	6.5

FOR RESEARCH USE ONLY

KAMIYA BIOMEDICAL COMPANY
12779 Gateway Drive, Seattle, WA 98168
Tel: (206) 575-8068 Fax: (206) 575-8094
Email: LifeScience@k-assay.com
www.k-assay.com