

Human TNFRSF18/GITR ELISA Kit

CATALOG NO: IRKTAH5270

LOT NO: SAMPLE

INTENDED USE

For quantitative detection of human TNFRSF18 in cell culture supernates, serum and plasma (heparin, EDTA).

BACKGROUND

Tumor necrosis factor receptor superfamily member 18 (TNFRSF18), also called GITR or AITR is a protein that in humans is encoded by the TNFRSF18 gene. This gene is mapped to 1p36.33. This gene encodes a member of the TNF-receptor superfamily. The encoded receptor has been shown to have increased expression upon T-cell activation, and it is thought to play a key role in dominant immunological self-tolerance maintained by CD25(+)CD4(+) regulatory T cells. Knockout studies in mice also suggest the role of this receptor is in the regulation of CD3-driven T-cell activation and programmed cell death. Three alternatively spliced transcript variants of this gene encoding distinct isoforms have been reported.

PRINCIPLE OF THE ASSAY

The human TNFRSF18 ELISA Kit was based on standard sandwich enzyme-linked immune-sorbent assay technology. A monoclonal antibody from mouse specific for TNFRSF18 has been precoated onto 96-well plates. Standards and test samples are added to the wells, a biotinylated detection polyclonal antibody from goat specific for TNFRSF18 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 TNFRSF18 amount of sample captured in plate.

REAGENTS

Description	Quantity
96-well plate precoated with anti- human TNFRSF18	1
Lyophilized recombinant human TNFRSF18 standard	10ng/tubex2
Biotinylated anti- human TNFRSF18 antibody	130µl(dilution 1:100)
Avidin-Biotin-Peroxidase Complex (ABC)	130µl(dilution 1:100)
Sample diluent buffer	30 ml
Antibody diluent buffer	12ml
ABC diluent buffer	12ml
TMB color developing agent	10ml
TMB stop solution	10ml

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 1.2g Tris, 8.5g NaCl; 450µl of purified acetic acid or 700µl of concentrated hydrochloric acid to 1000ml H₂O 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 Na₂HPO₄ and 0.2g NaH₂PO₄ to 1000ml distilled water and adjust pH to 7.2-7.6. Finally, adjust the total volume to 1L.

PROCEDURAL NOTES

1. To inspect the validity of experiment operation and the appropriateness of sample dilution proportion, pilot experiment using standards 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 standard 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°C for 30 min before using.

SAMPLE PREPARATION AND STORAGE

Store samples to be assayed within 24 hours at 2-8°C. For long-term storage, aliquot and freeze samples at -20°C. Avoid repeated freeze-thaw cycles.

- **Cell culture supernates:** Remove particulates by centrifugation, assay immediately or aliquot and store samples at -20°C.
- **Serum:** Allow the serum to clot in a serum separator tube (about 4 hours) at room temperature. Centrifuge at approximately 1000 X g for 15 min. Analyze the serum immediately or aliquot and store samples 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.

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 standard 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 (40-400ng/ml).** The working dilution is 1:100. i.e. Add 3 µl sample into 297 µl sample diluent buffer.
- **Medium target protein concentration (4-40ng/ml).** The working dilution is 1:10. i.e. Add 25 µl sample into 225 µl sample diluent buffer.
- **Low target protein concentration (62.5-4000pg/ml).** The working dilution is 1:2. i.e. Add 100 µl sample to 100 µl sample diluent buffer.
- **Very Low target protein concentration (≤62.5pg/ml).** No dilution necessary, or the working dilution is 1:2.

REAGENT PREPARATION AND STORAGE

- A. Reconstitution of the human TNFRSF18 standard : TNFRSF18 standard solution should be prepared no more than 2 hours prior to the experiment. Two tubes of TNFRSF18 standard (10ng per tube) are included in each kit. Use one tube for each experiment.
- 10,000pg/ml of human TNFRSF18 standard solution: Add 1 ml sample diluent buffer into one tube, keep the tube at room temperature for 10 min and mix thoroughly.
 - 4000pg/ml of human TNFRSF18 standard solution: Add 0.4ml of the above 10ng/ml TNFRSF18 standard solution into 0.6ml sample diluent buffer and mix thoroughly.
 - 2000pg/ml→62.5pg/ml of human TNFRSF18 standard solutions: Label 6 Eppendorf tubes with 2000pg/ml, 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml respectively. Aliquot 0.3ml of the sample diluent buffer into each tube. Add 0.3ml of the above 4000pg/ml TNFRSF18 standard solution into 1st tube and mix. Transfer 0.3 ml from 1st tube to 2nd tube and mix. Transfer 0.3ml from 2nd tube to 3rd tube and mix, and so on.
- Note:** The standard solutions are best used within 2 hours. The 10ng/ml standard solution should be stored at 4°C for up to 12 hours, or at -20°C for up to 48 hours. Avoid repeated freeze-thaw cycles.
- B. Preparation of biotinylated anti-human TNFRSF18 antibody working solution: The solution should be prepared no more than 2 hours prior to the experiment.
- The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)
 - Biotinylated anti-human TNFRSF18 antibody should be diluted in 1:100 with the antibody diluent buffer and mixed thoroughly. (i.e. Add 1µl Biotinylated anti-human TNFRSF18 antibody to 99µl 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.
- The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)
 - Avidin- Biotin-Peroxidase Complex (ABC) should be diluted in 1:100 with the ABC dilution buffer and mixed thoroughly. (i.e. Add 1µl ABC to 99µl ABC diluent buffer.)

ASSAY PROCEDURE

The ABC working solution and TMB color developing agent must be kept warm at 37°C for 30 min before use. When diluting samples and reagents, they must be mixed completely and evenly. Standard TNFRSF18 detection curve should be prepared for each experiment. The user will decide sample dilution fold by crude estimation of TNFRSF18 amount in samples.

1. Aliquot 0.1ml per well of the 4000pg/ml, 2000pg/ml, 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml human TNFRSF18 standard solutions into the precoated 96-well plate. Add 0.1ml of the sample diluent buffer into the control well (Zero well). Add 0.1ml of each properly diluted sample of human cell culture supernates, serum or plasma (heparin, EDTA) to each empty well. **See "Sample Dilution Guideline" above for details.** It is recommended that each human TNFRSF18 standard solution and each sample be measured in duplicate.
2. Seal the plate with the cover and incubate at 37°C 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 0.1ml of biotinylated anti-human TNFRSF18 antibody working solution into each well and incubate the plate at 37°C 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 0.1ml of prepared ABC working solution into each well and incubate the plate at 37°C 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 90µl of prepared TMB color developing agent into each well and incubate plate at 37°C in dark for 25-30 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 TNFRSF18 standard solutions; the other wells show no obvious color).
9. Add 0.1ml of prepared TMB stop solution into each well. The color changes into yellow immediately.
10. Read the O.D. absorbance at 450nm 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 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 human TNFRSF18 concentration of the samples can be interpolated from the standard curve. **Note:** if the samples measured were diluted, multiply the dilution factor to the concentrations from interpolation to obtain the concentration before dilution.

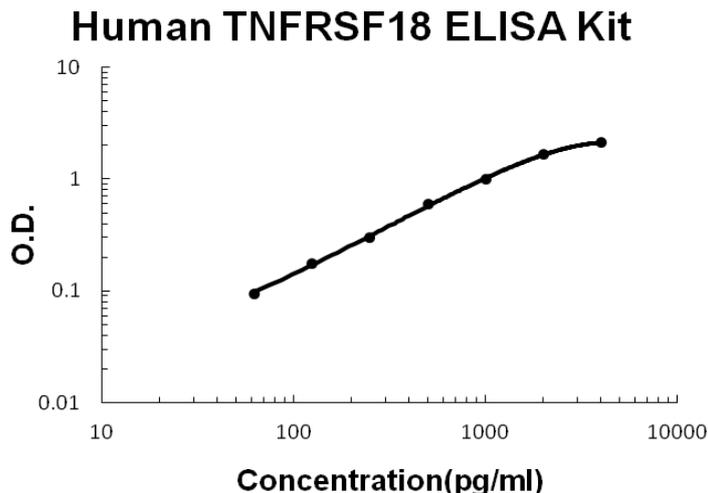
TYPICAL DATA OBTAINED FROM HUMAN TNFRSF18

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

Concentration(pg/ml)	0.0	62.5	125	250	500	1000	2000	4000
O.D	0.031	0.095	0.175	0.299	0.596	1.008	1.651	2.111

TYPICAL HUMAN TNFRSF18 ELISA KIT STANDARD CURVE

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



Range	62.5pg/ml-4000pg/ml
Sensitivity	< 10pg/ml
Specificity	Natural and recombinant human TNFRSF18
Cross-reactivity	No detectable cross-reactivity with other relevant proteins

STORAGE

Store at 4°C for 6 months, at -20°C for 12 months. Avoid multiple freeze-thaw cycles (Shipped with wet ice.)

PRECISION

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.

Sample	Intra-Assay Precision			Inter-Assay Precision		
	1	2	3	1	2	3
n	16	16	16	24	24	24
Mean(pg/ml)	342	1565	3014	540	1896	3242
Standard deviation	19.84	62.6	105.5	36.18	110	243.2
CV(%)	5.8	4	3.5	6.7	5.8	7.5

SUMMARY

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

REFERENCES

1. Grohmann, U., Volpi, C., Fallarino, F., Bozza, S., Bianchi, R., Vacca, C., Orabona, C., Belladonna, M. L., Ayroldi, E., Nocentini, G., Boon, L., Bistoni, F., Fioretti, M. C., Romani, L., Riccardi, C., Puccetti, P. Reverse signaling through GITR ligand enables dexamethasone to activate IDO in allergy. *Nature Med.* 13: 579-586, 2007.
2. Ji, H., Liao, G., Faubion, W. A., Abadia-Molina, A. C., Cozzo, C., Laroux, F. S., Caton, A., Terhorst, C. Cutting edge: the natural ligand for glucocorticoid-induced TNF receptor-related protein abrogates regulatory T cell suppression. *J. Immun.* 172: 5823-5827, 2004.

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