



# TNFRSF18 (Human) ELISA Kit

Catalog Number KA1719

96 assays

Version: 02

Intended for research use only

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## **Introduction**

### **Background**

GITR (glucocorticoid induced tumor necrosis factor receptor family related gene) is a type-1 transmembrane protein of 228 amino acids belonging to the TNF and NGF receptor family of proteins. GITR is expressed in normal T-lymphocytes from thymus, spleen. Constitutive expression of a transfected GITR gene induces resistance to apoptosis induced by anti CD3 monoclonal antibodies. The human homolog of GITR is expressed in lymph node and peripheral blood leukocytes. Its expression is up-regulated in human peripheral mononuclear cells mainly after stimulation with antibodies against CD3 and CD28.

### **Principle of the Assay**

TNFRSF18 (Human) ELISA Kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of human GITR in serum, plasma, cell culture supernatants and urine. This assay employs an antibody specific for human GITR coated on a 96-well plate. Standards and samples are pipetted into the wells and GITR present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-human GITR antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of GITR bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

## General Information

### Materials Supplied

Component	Amount
GITR Microplate (Item A): Coated with anti-human GITR.	96 (8x12) wells
Wash Buffer Concentrate (20x) (Item B): 20x concentrated solution.	25 mL
Standards (Item C): Recombinant human GITR.	2 vials
Assay Diluent A (Item D): For Standard/Sample (serum/plasma) diluent. 0.09% sodium azide as preservative.	30 mL
Assay Diluent B (Item E): 5x concentrated buffer. For Standard/Sample (cell culture medium/urine) diluent.	15 mL
Detection Antibody GITR (Item F): Biotinylated anti-human GITR (each vial is enough to assay half microplate).	2 vials
HRP-Streptavidin Concentrate (Item G): 400x concentrated HRP-conjugated streptavidin.	200 $\mu$ L
TMB One-Step Substrate Reagent (Item H): 3,3',5,5'-tetramethylbenzidine (TMB) in buffered solution.	12 mL
Stop Solution (Item I): 0.2 M sulfuric acid.	8 mL

### Storage Instruction

May be stored for up to 6 months at 2 to 8°C from the date of shipment. Standard (recombinant protein) should be stored at -20°C or -80°C (recommended at -80°C) after reconstitution. Opened Microplate Wells or reagents may be store for up to 1 month at 2 to 8°C. Return unused wells to the pouch containing desiccant pack, reseal along entire edge.

*Note: the kit can be used within one year if the whole kit is stored at -20°C. Avoid repeated freeze-thaw cycles.*

### Materials Required but Not Supplied

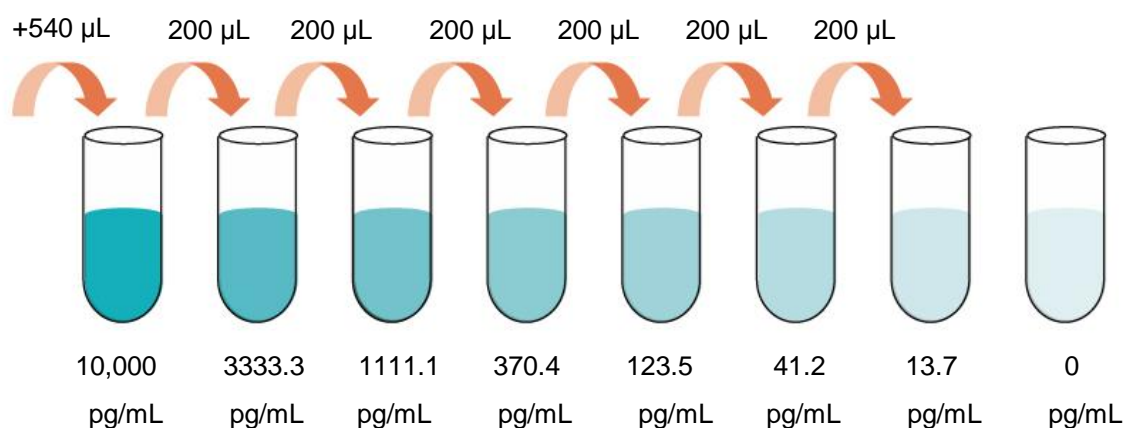
- ✓ Microplate reader capable of measuring absorbance at 450 nm.
- ✓ Precision pipettes to deliver 2  $\mu$ L to 1 mL volumes.
- ✓ Adjustable 1-25 mL pipettes for reagent preparation.
- ✓ 100 mL and 1 liter graduated cylinders.
- ✓ Absorbent paper.
- ✓ Distilled or deionized water.
- ✓ Log-log graph paper or computer and software for ELISA data analysis.
- ✓ Tubes to prepare standard or sample dilutions.

## Assay Protocol

### Reagent Preparation

1. Bring all reagents and samples to room temperature (18 - 25°C) before use.
2. Assay Diluent B should be diluted 5-fold with deionized or distilled water.
3. Preparation of standard: Briefly spin the vial of Item C and then add 400  $\mu$ L Assay Diluent A (for serum/plasma samples) or 1x Assay Diluent B (for cell culture medium and urine, Assay Diluent B should be diluted 5-fold with deionized or distilled water before use) into Item C vial to prepare a 100 ng/mL standard. Dissolve the powder thoroughly by a gentle mix. Add 60  $\mu$ L GITR standard from the vial of Item C, into a tube with 540  $\mu$ L Assay Diluent A or 1x Assay Diluent B to prepare a 10,000 pg/mL stock standard solution. Pipette 400  $\mu$ L Assay Diluent A or 1x Assay Diluent B into each tube. Use the stock standard solution to produce a dilution series (shown below). Mix each tube thoroughly before the next transfer. Assay Diluent A or 1x Assay Diluent B serves as the zero standard (0 pg/mL).

60  $\mu$ L standard



4. If the Wash Concentrate (20x) (Item B) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to yield 400 mL of 1x Wash Buffer.
5. Briefly spin the Detection Antibody vial (Item F) before use. Add 100  $\mu$ L of 1x Assay Diluent B into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4°C for 5 days). The detection antibody concentrate should be diluted 80-fold with 1x Assay Diluent B and used in step 4 of Assay Procedure.
6. Briefly spin the HRP-Streptavidin concentrate vial (Item G) and pipette up and down to mix gently before use. HRP-Streptavidin concentrate should be diluted 400-fold with 1x Assay Diluent B.  
For example: Briefly spin the vial (Item G) and pipette up and down to mix gently. Add 35  $\mu$ L of HRP-Streptavidin concentrate into a tube with 14 mL 1x Assay Diluent B to prepare a final 400 fold diluted HRP-Streptavidin solution (don't store the diluted solution for next day use). Mix well.

### **Sample Preparation**

Sample dilution: If your samples need to be diluted, Assay Diluent A (Item D) should be used for dilution of serum/plasma samples. 1x Assay Diluent B (Item E) should be used for dilution of culture supernatants and urine.

*Note: Suggested dilution for normal serum/plasma: 2 fold\**

\*Please note that levels of the target protein may vary between different specimens. Optimal dilution factors for each sample must be determined by the investigator.

### **Assay Procedure**

1. Bring all reagents and samples to room temperature (18 - 25°C) before use. It is recommended that all standards and samples be run at least in duplicate.
2. Add 100 µL of each standard (see Reagent Preparation step 3) and sample into appropriate wells. Cover well and incubate for 2.5 hours at room temperature or over night at 4°C with gentle shaking.
3. Discard the solution and wash 4 times with 1x Wash Solution. Wash by filling each well with Wash Buffer (300 µL) using a multi-channel Pipette or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
4. Add 100 µL of 1x prepared biotinylated antibody (Reagent Preparation step 5) to each well. Incubate for 1 hour at room temperature with gentle shaking.
5. Discard the solution. Repeat the wash as in step 3.
6. Add 100 µL of prepared Streptavidin solution (see Reagent Preparation step 6) to each well. Incubate for 45 minutes at room temperature with gentle shaking.
7. Discard the solution. Repeat the wash as in step 3.
8. Add 100 µL of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking.
9. Add 50 µL of Stop Solution (Item I) to each well. Read at 450 nm immediately.

#### ✓ Summary

1. Prepare all reagents, samples and standards as instructed.
2. Add 100 µL standard or sample to each well. Incubate 2.5 hours at room temperature or over night at 4°C.
3. Add 100 µL prepared biotin antibody to each well. Incubate 1 hour at room temperature.
4. Add 100 µL prepared Streptavidin solution. Incubate 45 minutes at room temperature.
5. Add 100 µL TMB One-Step Substrate Reagent to each well. Incubate 30 minutes at room temperature.
6. Add 50 µL Stop Solution to each well. Read at 450 nm immediately.

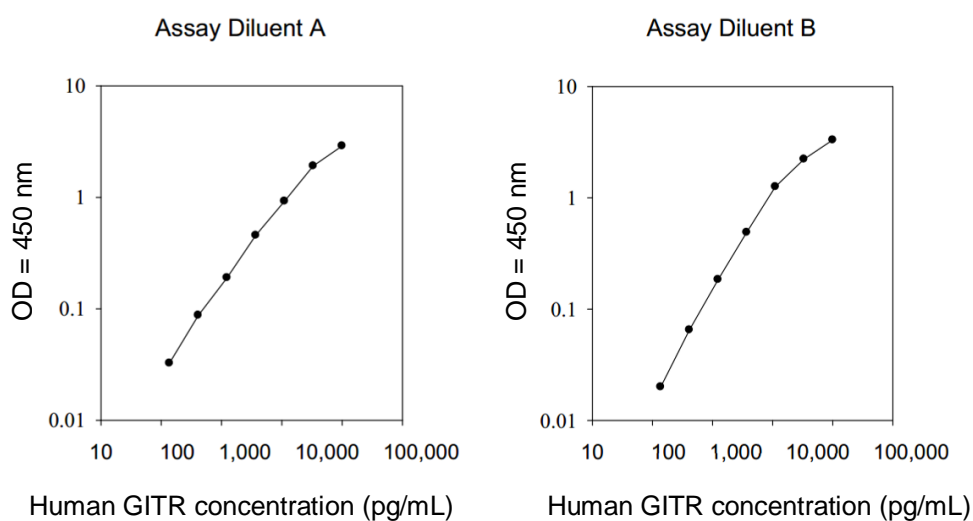
## Data Analysis

### Calculation of Results

Calculate the mean absorbance for each set of duplicate standards, controls and samples, and subtract the average zero standard optical density. Plot the standard curve on log-log graph paper or using Sigma plot software, with standard concentration on the x-axis and absorbance on the y-axis. Draw the best-fit straight line through the standard points.

#### ✓ Typical Data

These standard curves are for demonstration only. A standard curve must be run with each assay.



### Performance Characteristics

#### ✓ Sensitivity

The minimum detectable dose of GTR is typically less than 15 pg/mL.

#### ✓ Recovery

Recovery was determined by spiking various levels of recombinant human GTR into normal human serum, plasma and cell culture media. Mean recoveries are as follows:

Sample Type	Average % Recovery	Range (%)
Serum	94.18	83-104
Plasma	92.75	82-102
Cell culture media	95.69	84-105

✓ Linearity

Sample Type		Serum	Plasma	Cell Culture Media
1:2	Average % of Expected	89	90	95
	Range (%)	82-102	83-103	84-104
1:4	Average % of Expected	86	85	89
	Range (%)	82-101	82-102	83-102
1:8	Average % of Expected	84	86	92
	Range (%)	81-101	82-102	83-103

✓ Reproducibility

Intra-Assay: CV<10%

Inter-Assay: CV<12%

✓ Specificity

Cross Reactivity: This ELISA kit shows no cross-reactivity with any of the cytokines tested: human Angiogenin, BDNF, BLC, ENA-78, FGF-4, IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-3, IL-4, IL-5, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12 p70, IL-12 p40, IL-13, IL-15, IL-309, IP-10, G-CSF, GM-CSF, IFN- $\gamma$ , Leptin, MCP-1, MCP-2, MCP-3, MDC, MIP-1 $\alpha$ , MIP-1  $\beta$ , MIP-1 $\delta$ , PARC, PDGF, RANTES, SCF, TARC, TGF- $\beta$ , TIMP-1, TIMP-2, TNF- $\alpha$ , TNF- $\beta$ , TPO, VEGF.

## Resources

### Troubleshooting

Problem	Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standard dilution	Ensure a brief spin of Item C and dissolve the powder thoroughly by a gentle mix.
Low signal	Too brief incubation times	Ensure sufficient incubation time; assay procedure step 2 may change to over night
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Large CV	Inaccurate pipetting	Check pipettes
High background	Plate is insufficiently washed	Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed.
	Contaminated wash buffer	Make fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	Store your standard at $\leq -20^{\circ}\text{C}$ after reconstitution, others at $4^{\circ}\text{C}$ . Keep substrate solution protected from light
	Stop solution	Stop solution should be added to each well before measure

### References

1. Gurney AL et al. Identification of a new member of the tumor necrosis factor family and its receptor, a human ortholog of mouse GITR. *Current Biology* 9(4): 215-8 (1999).
2. Kwon B et al. Identification of a novel activation-inducible protein of the tumor necrosis factor receptor superfamily and its ligand. *Journal of Biological Chemistry* 274(10): 6056-61 (1999).
3. Nocentini G et al. A new member of the tumor necrosis factor nerve growth factor receptor family inhibits t cell receptor induced apoptosis. *Proceedings of the National Academy of Science (USA)* 94(12): 6216-6221 (1997).

**Plate Layout**

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