Human GITR/TNFRSF18 Sandwich ELISA Kit Datasheet

Please read it entirely before use

Catalogue Number: KE00220

Size: 5*96T

Sensitivity: 11.5 pg/mL

Range: 62.5-4000 pg/mL

Usage: For the quantitative detection of human GITR/TNFRSF18 concentrations in serum, plasma and cell culture supernatant.

This product is for research use only and not for use in human or animal therapeutic or diagnostic.

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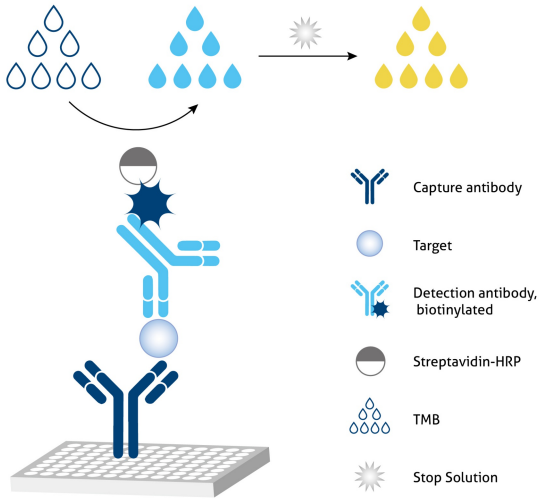
1. Background

Glucocorticoid-induced TNFR-related protein (GITR), also known as CD357 or TNFRSF18, is a member of the tumor necrosis factor receptor (TNF-R) superfamily. GITR is expressed constitutively at high levels in T regulatory cells (Treg cells) and plays a key role in dominant immunological self-tolerance maintained by CD25+CD4+ regulatory T cells. It is expressed at low levels on resting responder T cells. The expression of GITR on T cells can be upregulated upon activation. GITR is activated by GITR ligand (GITRL) which is mainly expressed on APC. GITR-GITRL interactions could co-stimulate both responder T-cell functions and the suppressive functions of Treg cells.

2. Principle



Sandwich ELISA structure (Detection antibody labeled with biotin)

A capture antibody is pre-coated onto the bottom of wells which binds to analyte of interest. A detection antibody labeled with biotin also binds to the analyte. Streptavidin-HRP binds to the biotin. TMB acts as the HRP substrate and the solution color will change from colorless to blue. A stop solution containing sulfuric acid turns solution yellow. The color intensity is proportional to the quantity of bound protein which is measurable at 450 nm with the correction wavelength set at 630 nm.

3. Required Materials

3.1 A microplate reader capable of measuring absorbance at 450 nm with the correction wavelength set at 630 nm.

3.2 Calibrated, adjustable precision pipettes and disposable plastic tips. A manifold multi-channel pipette is recommended for large assays.

3.3 Plate washer: automated or manual.

3.4 Absorbent paper towels.

3.5 Glass or plastic tubes to prepare standard and sample dilutions.

3.6 Beakers and graduated cylinders.

3.7 Log-log or semi-log graph paper or computer and software for ELISA data analysis. A four-parameter logistic (4-PL) curve-fit is recommended.

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4. Kit Components and Storage

Microplate - antibody coated 96-well microplate (8 well × 12 strips)	5 plates	**Unopened Kit:** Store at 2-8°C for 6 months or -20°C for 12 months. **Opened Kit:** All reagents stored at 2-8°C for 7 days. **Please use a new standard for each assay.**
Protein standard - 8000 pg/bottle; lyophilized	10 bottles	
Detection Antibody, biotinylated (100×) - 600 μL/vial*	1 vial	
Streptavidin-horseradish peroxidase (HRP) (100×) - 600 μL/vial*	1 vial	
Sample Diluent PT 5 - 150 mL/bottle. For serum	1 bottle	
Sample Diluent PT 3-eg - 150 mL/bottle. For plasma	1 bottle	
Sample Diluent PT 1 - 150 mL/bottle. For cell culture supernatant	1 bottle	
Detection Diluent - 150 mL/bottle	1 bottle	
Wash Buffer Concentrate (20×) - 150 mL/bottle	1 bottle	
Tetramethylbenzidine Substrate (TMB) - 60 mL/bottle	1 bottle	
Stop Solution - 60 mL/bottle	1 bottle	
Plate Cover Seals	15 pieces	

* Centrifugation immediately before use

5. Safety Notes

5.1 Avoid any skin and eye contact with Stop Solution and TMB. In case of contact, wash thoroughly with water.

5.2 Do not use the kit after the expiration date.

5.3 Do not mix or substitute reagents or materials from other kit lots or other sources.

5.4 Be sure to wear protective equipment such as gloves, masks and goggles during the experiment.

5.5 When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer to improve assay precision

6. Sample Collection and Storage

6.1 Serum: Allow blood samples to clot for 30 minutes, followed by centrifugation for 15 minutes at 1000xg. Clear serum can be assayed immediately or aliquoted and stored at -20℃. Avoid repeated freeze-thaw cycles.

6.2 Plasma: Use EDTA, heparin, or citrate as an anticoagulant for plasma collection. Centrifuge for 15 minutes at 1000xg within 30 minutes of collection. The plasma can be assayed immediately or aliquoted and stored at -20℃. Avoid repeated freeze-thaw cycles.

6.3 Cell Culture Supernatant: Remove particulates by centrifugation for 5 minutes at 500xg and assay immediately or aliquot and store samples at ≤ -20℃. Avoid repeated freeze-thaw cycles.

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7. Regent Preparation

7.1 Wash Buffer (1X): If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 30 mL of Wash Buffer Concentrate(20X) to 570 mL deionized or distilled water to prepare 1X Wash Buffer.

7.2 Detection Antibody (1X): Dilute 100X Detection Antibody 1:100 using Detection Diluent prior to assay. Suggested 1:100 dilution: 10 μL 100X Detection Antibody + 990 μL Detection Diluent (Centrifuge the 100 X Detection Antibody solution for a few seconds prior to use).

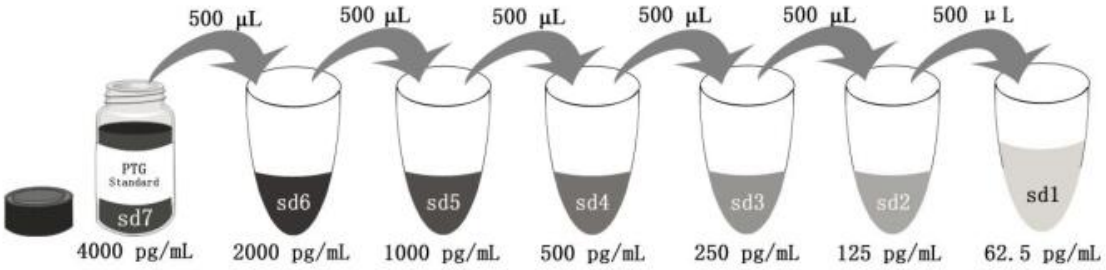
7.3 Streptavidin-HRP (1X): Dilute 100X Streptavidin-HRP 1:100 using Detection Diluent prior to assay. Suggested 1:100 dilution: 10 μL 100X Streptavidin-HRP + 990 μL Detection Diluent (Centrifuge the 100X Streptavidin-HRP solution for a few seconds prior to use).

7.4 Sample Dilution: Different samples should be diluted with corresponding Sample Diluent, samples may require further dilution if the readout values are higher than the highest standard OD reading. Variations in sample collection, processing and storage may affect the results of the measurement.

Recommended Dilution for different sample types: 1:2 is recommended for human serum and plasma; 1:2 is recommended for cell culture supernatant.

7.5 Standard Serial Dilution:

For human serum samples, add 2 mL Sample Diluent PT 5 in protein standard; For human plasma, add 2 mL Sample Diluent PT 3-eg in protein standard; For cell culture supernatant, add 2 mL Sample Diluent PT 1 in protein standard.



Add # μL of Standard diluted in the previous step	—	500 μL	500 μL	500 μL	500 μL	500 μL	500 μL
# μL of Sample Diluent **PT 5, PT 3-eg or PT 1**	**2000 μL**	500 μL	500 μL	500 μL	500 μL	500 μL	500 μL
	"sd7"	"sd6"	"sd5"	"sd4"	"sd3"	"sd2"	"sd1"

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8. Assay Procedure Summary

Bring all reagents to room temperature before use (Detection antibody and Streptavidin-HRP can be used immediately). To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.

8.1 Take out the required number of microplate strips and return excess strips to the foil pouch containing the drying reagent pack and reseal; store at 4°C immediately. Microplate strips should be used in one week.

8.2 Preset the layout of the microplate, including control group,standard group and sample group, add 100 μL of each standard and sample to the appropriate wells.(Make sure sample addition is uninterrupted and completed within 5 to 10 minutes, It is recommended to assay all standards, controls, and samples in duplicate).

8.3 Seal plate with cover seal, pressing it firmly onto top of microwells. Incubate the plate for 2 hours at 37°C.

8.4 Wash

1) Gently remove the cover seal. Discard the liquid from wells by aspirating or decanting. Remove any residual solution by tapping the plate a few times on fresh paper towels.

2) Wash 4 times with 1X Wash Buffer, using at least 350-400 μL per well. Following the last wash, firmly tap plates on fresh towels 10 times to remove residual Wash Buffer. Avoid getting any towel fibers in the wells or wells drying out completely.

8.5 Add 100 μL of 1X Detection Antibody solution (refer to Reagent Preparation7.2) to each well. Seal plate with cover seal and incubate for 1 hour at 37°C.

8.6 Repeat wash step in 8.4.

8.7 Add 100 μL of 1X Streptavidin-HRP solution (refer to Reagent Preparation7.3) to each well. Seal plate with cover seal and incubate the plate for 40 minutes at 37°C .

8.8 Repeat wash step in 8.4.

8.9 Signal development: Add 100 μL of TMB substrate solution to each well, protected from light. Incubate for 15 to 20 minutes. Substrate Solution should remain colorless until added to the plate.

8.10 Quenching color development: Add 100 μL of Stop Solution to each well in the same order as addition of the TMB substrate. Mix by tapping the side of the plate gently. NB: Avoid skin and eye contact with the Stop solution.

8.11 Read results: Immediately after adding Stop solution read the absorbance on a microplate reader at a wavelength of 450 nm. If possible, perform a double wavelength readout (450 nm and 630 nm).

8.12 Data analysis: Calculate the average of the duplicate readings (OD value) for each standard and sample, and subtract the average of the zero standard absorbance. Construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis, use four-parameter logistic curve- fit (4-PL) analysis to do this. If the samples have been diluted, the OD readout from the standard curve must be multiplied by the dilution factor used.

Step	Reagent	Volume	Incubation	Wash	Notes
1	Standard and Samples	100 µL	120 min	4 times	Cover Wells incubate at 37°C
2	Diluent Antibody Solution	100 µL	60 min	4 times	Cover Wells incubate at 37°C
3	Diluent HRP Solution	100 µL	40 min	4 times	Cover Wells incubate at 37°C
4	TMB Substrate	100 µL	15-20 min	Do not wash	Incubate in the dark at 37°C
5	Stop Solution	100 µL	0 min	Do not wash	-
6	Read plate at 450 nm and 630 nm immediately after adding Stop solution. DO NOT exceed 5 minutes.				

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9. Validation Data

9.1 Standard curve

These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

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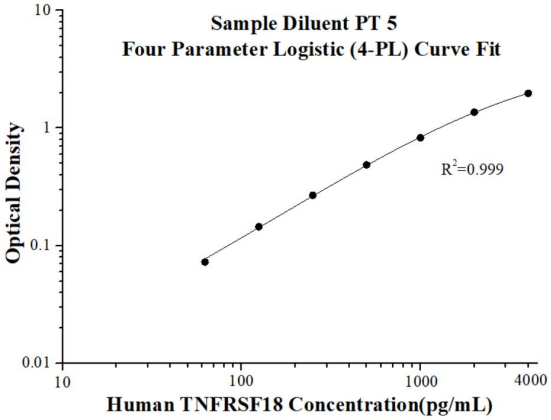
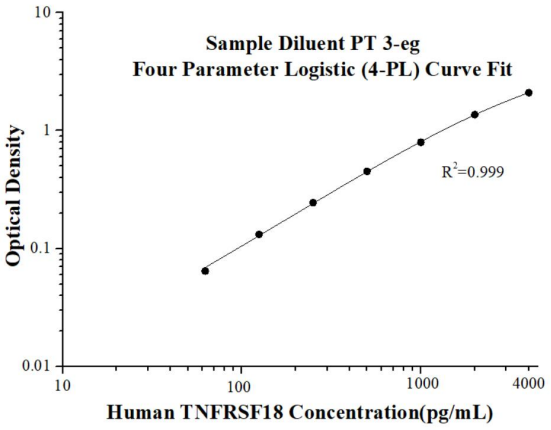
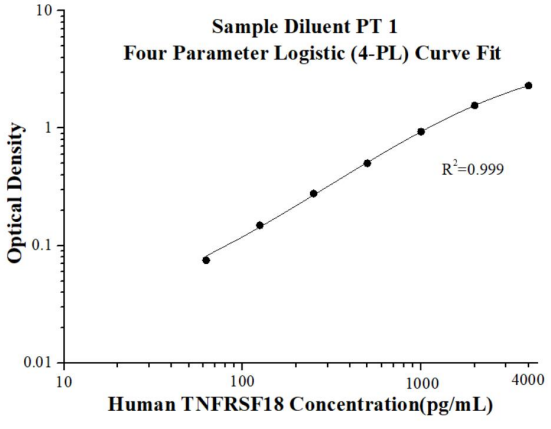


(pg/mL)	O.D	Average	Corrected
0	0.09 0.098	0.094	-
62.5	0.165 0.168	0.1665	0.0725
125	0.236 0.242	0.239	0.145
250	0.362 0.363	0.3625	0.2685
500	0.595 0.566	0.5805	0.4865
1000	0.917 0.927	0.922	0.828
2000	1.464 1.454	1.459	1.365
4000	2.095 2.044	2.0695	1.9755



(pg/mL)	O.D	Average	Corrected
0	0.068 0.068	0.068	-
62.5	0.131 0.134	0.1325	0.0645
125	0.199 0.201	0.200	0.132
250	0.312 0.314	0.313	0.245
500	0.528 0.512	0.520	0.452
1000	0.872 0.86	0.866	0.798
2000	1.467 1.405	1.436	1.368
4000	2.191 2.144	2.1675	2.0995



(pg/mL)	O.D	Average	Corrected
0	0.104 0.103	0.1035	-
62.5	0.181 0.176	0.1785	0.075
125	0.253 0.252	0.2525	0.149
250	0.376 0.386	0.381	0.2775
500	0.608 0.604	0.606	0.5025
1000	1.024 1.053	1.0385	0.935
2000	1.717 1.617	1.667	1.5635
4000	2.403 2.41	2.4065	2.303

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9.2 Precision

Intra-assay Precision (Precision within an assay) Three samples of known concentration were tested 20 times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays) Three samples of known concentration were tested in 24 separate assays to assess inter-assay precision.

Intra-assay Precision				
Sample	n	Mean (pg/mL)	SD	CV%
1	20	165.7	2.2	1.3
2	20	663.9	13.3	2.0
3	20	2,812.0	99.6	3.5

Inter-assay Precision				
Sample	n	Mean (pg/mL)	SD	CV%
1	24	169.7	7.2	4.3
2	24	694.1	19.8	2.8
3	24	2,957.6	107.8	3.6

9.3 Recovery

The recovery of human TNFRSF18 spiked to three different levels throughout the range of the assay in various matrices was evaluated.

Sample Type		Average% of Expected	Range (%)
Human serum	1:2	84	75-97
	1:4	82	74-89
Human plasma	1:2	88	75-112
	1:4	97	84-112
Cell culture supernatant	1:2	108	95-124
	1:4	112	97-124

9.4 Sample values

Human serum, plasma samples from volunteers were evaluated for human TNFRSF18 in this assay. No medical histories were available for the donors used in this study.

Sample Type	Range (pg/mL)	Mean (pg/mL)
Human serum (n=24)	42.6-417.9	174.9
Human plasma (n=22)	25.3-655.8	278.9

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9.5 Sensitivity

The minimum detectable dose of human TNFRSF18 is 11.5 pg/mL. This was determined by adding two standard deviations to the concentration corresponding to the mean O.D. of 20 zero standard replicates.

9.6 Linearity

To assess the linearity of the assay, human serum, plasma and cell culture supernatant were spiked with high concentrations of TNFRSF18 in various matrices and diluted with the appropriate **Sample Diluent** to produce samples with values within the dynamic range of the assay.

Sample Type		Range (%)	Average% of Expected
Human serum (Sample Diluent PT 5)	1:2	76-105	87
	1:4	77-111	93
	1:8	92-127	108
	1:16	106-119	113
Human plasma (Sample Diluent PT 3-eg)	1:2	79-89	86
	1:4	87-107	98
	1:8	93-114	102
	1:16	88-123	103
Cell culture supernatant (Sample Diluent PT 1)	1:2	85-104	95
	1:4	89-111	100
	1:8	88-116	102
	1:16	88-118	103

10. References

1. Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S. Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. Nat Immunol. 2002;3(2):135-142.

2. Nocentini G, Riccardi C. GITR: a multifaceted regulator of immunity belonging to the tumor necrosis factor receptor superfamily. Eur J Immunol. 2005;35(4):1016-1022.

3. Shevach EM, Stephens GL. The GITR-GITRL interaction: co-stimulation or contrasuppression of regulatory activity? Nat Rev Immunol. 2006 Aug;6(8):613-8.

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