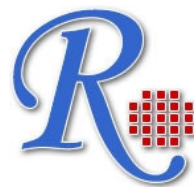


# **RayBio® Human GITR ELISA Kit**

**User Manual  
(Revised Feb 1, 2009)**

**RayBio® Human GITR ELISA  
Kit Protocol**

(Cat#: ELH-GITR-001)



**RayBiotech, Inc.**

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**RayBio® Human GTR  
ELISA Kit Protocol**

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## I. INTRODUCTION

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.

The RayBio® Human GITR ELISA (Enzyme-Linked Immunosorbent Assay) 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.

## II. REAGENTS

1. GITR Microplate (Item A): 96 wells (12 strips x 8 wells) coated with anti-human GITR.
2. Wash Buffer Concentrate (20x) (Item B): 25 ml of 20x concentrated solution.
3. Standards (Item C): 2 vials, recombinant human GITR.

4. Assay Diluent A (Item D): 30 ml, 0.09% sodium azide as preservative. For Standard/Sample (serum/plasma) diluent.
5. Assay Diluent B (Item E): 15 ml of 5x concentrated buffer. For Standard/Sample (cell culture medium/urine) diluent.
6. Detection Antibody GITR (Item F): 2 vial of biotinylated anti-human GITR (each vial is enough to assay half microplate).
7. HRP-Streptavidin Concentrate (Item G): 8  $\mu$ l of 20,000x concentrated HRP-conjugated streptavidin.
8. TMB One-Step Substrate Reagent (Item H): 12 ml of 3,3',5,5'-tetramethylbenzidine (TMB) in buffered solution.
9. Stop Solution (Item I): 8 ml of 2 M sulfuric acid.

### **III. STORAGE**

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.

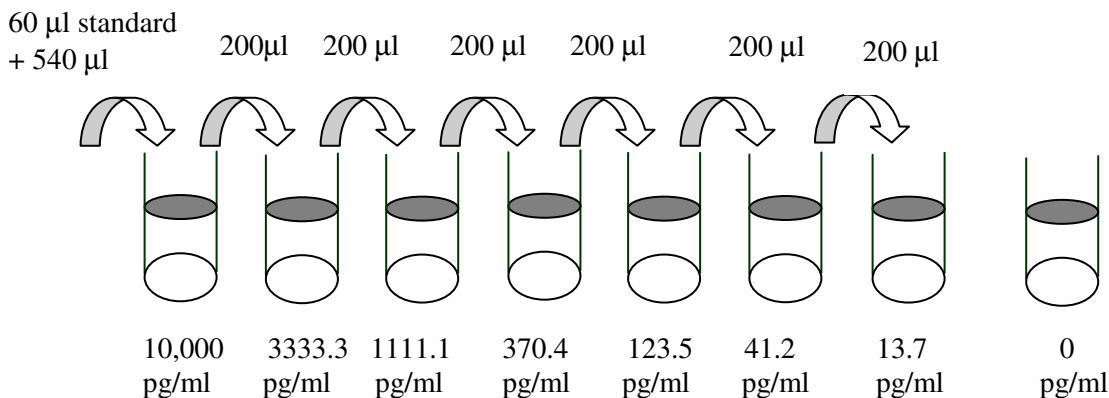
Note: the kit can be used within one year if the whole kit is stored at -20 °C . Avoid repeated freeze-thaw cycles.

### **IV. ADDITIONAL MATERIALS REQUIRED**

- 1 Microplate reader capable of measuring absorbance at 450 nm.
- 2 Precision pipettes to deliver 2  $\mu$ l to 1 ml volumes.
- 3 Adjustable 1-25 ml pipettes for reagent preparation.
- 4 100 ml and 1 liter graduated cylinders.
- 5 Absorbent paper.
- 6 Distilled or deionized water.
- 7 Log-log graph paper or computer and software for ELISA data analysis.
- 8 Tubes to prepare standard or sample dilutions.

## V. REAGENT PREPARATION

1. Bring all reagents and samples to room temperature (18 - 25°C) before use.
2. Sample dilution: If your samples need to be diluted, Assay Diluent A (Item D) is used for dilution of serum/plasma samples, and Assay Diluent B (Item E) is used for dilution of culture supernatants and urine.
3. Assay Diluent B should be diluted 5-fold with deionized or distilled water.
4. Preparation of standard: **Briefly spin the vial of Item C** and then add 400 µl 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 µl GITR standard from the vial of Item C, into a tube with 540 µl Assay Diluent A or 1x Assay Diluent B to prepare a 10,000 pg/ml stock standard solution. Pipette 400 µl 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).



5. 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.
6. 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 Part VI Assay Procedure.
7. 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 20,000-fold with 1x Assay Diluent B.

*For example: Briefly spin the vial (Item G) and pipette up and down to mix gently . Add 2  $\mu$ l of HRP-Streptavidin concentrate into a tube with 198.0  $\mu$ l 1x Assay Diluent B to prepare a 100-fold diluted HRP-Streptavidin solution (don't store the diluted solution for next day use). Mix through and then pipette 70  $\mu$ l of prepared 100-fold diluted solution into a tube with 14 ml 1x Assay Diluent B to prepare a final 20,000 fold diluted HRP-Streptavidin solution.*

## **VI. 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 2) 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 6) 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 7) 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.

## **VII. ASSAY PROCEDURE SUMMARY**

1. Prepare all reagents, samples and standards as instructed.



2. Add 100  $\mu$ l standard or sample to each well.  
Incubate 2.5 hours at room temperature or over night at 4°C.



3. Add 100  $\mu$ l prepared biotin antibody to each well.  
Incubate 1 hour at room temperature.



4. Add 100  $\mu$ l prepared Streptavidin solution.  
Incubate 45 minutes at room temperature.



5. Add 100  $\mu$ l TMB One-Step Substrate Reagent to each well.  
Incubate 30 minutes at room temperature.



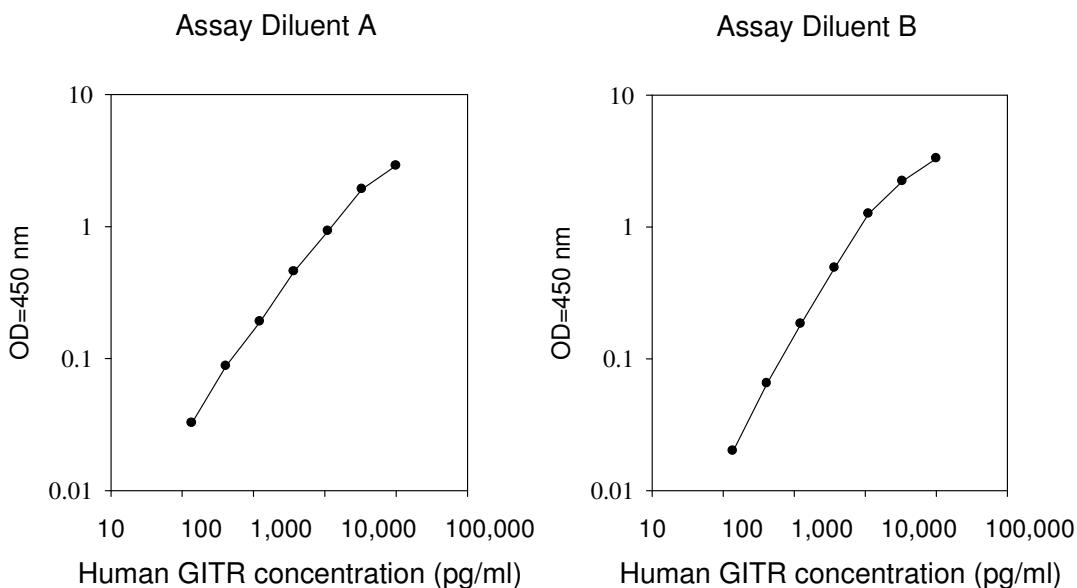
6. Add 50  $\mu$ l Stop Solution to each well.  
Read at 450 nm immediately.

## **VIII. 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.

## A. TYPICAL DATA

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



## B. SENSITIVITY

The minimum detectable dose of GITR is typically less than 15 pg/ml.

## C. RECOVERY

Recovery was determined by spiking various levels of recombinant human GITR 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

## D. LINEARITY

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

## E. REPRODUCIBILITY

Intra-Assay: CV<10%

Inter-Assay: CV<12%

## IX. SPECIFICITY

Cross Reactivity: This ELISA kit shows no cross-reactivity with any of the cytokines tested (*e.g.*, 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).

## **X. 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).

## XI. TROUBLESHOOTING GUIDE

<b>Problem</b>	<b>Cause</b>	<b>Solution</b>
1. Poor standard curve	<ol style="list-style-type: none"> <li>1. Inaccurate pipetting</li> <li>2. Improper standard dilution</li> </ol>	<ol style="list-style-type: none"> <li>1. Check pipettes</li> <li>2. Ensure a brief spin of Item C and dissolve the powder thoroughly by a gentle mix.</li> </ol>
2. Low signal	<ol style="list-style-type: none"> <li>1. Too brief incubation times</li> <li>2. Inadequate reagent volumes or improper dilution</li> </ol>	<ol style="list-style-type: none"> <li>1. Ensure sufficient incubation time; assay procedure step 2 may change to over night</li> <li>2. Check pipettes and ensure correct preparation</li> </ol>
3. Large CV	<ol style="list-style-type: none"> <li>1. Inaccurate pipetting</li> </ol>	<ol style="list-style-type: none"> <li>1. Check pipettes</li> </ol>
4. High background	<ol style="list-style-type: none"> <li>1. Plate is insufficiently washed</li> <li>2. Contaminated wash buffer</li> </ol>	<ol style="list-style-type: none"> <li>1. Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed.</li> <li>2. Make fresh wash buffer</li> </ol>
5. Low sensitivity	<ol style="list-style-type: none"> <li>1. Improper storage of the ELISA kit</li> <li>2. Stop solution</li> </ol>	<ol style="list-style-type: none"> <li>1. Store your standard at <math>&lt;-20^{\circ}\text{C}</math> after reconstitution, others at <math>4^{\circ}\text{C}</math>. Keep substrate solution protected from light</li> <li>2. Stop solution should be added to each well before measure</li> </ol>

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