

# Mouse TNFRSF9/4-1BB ELISA Kit

Catalog No. EA100673

Size 96T(8x12 divisible strips)

**For quantitative detection of mouse TNFRSF9 in cell culture supernates, serum and plasma(heparin, EDTA).**

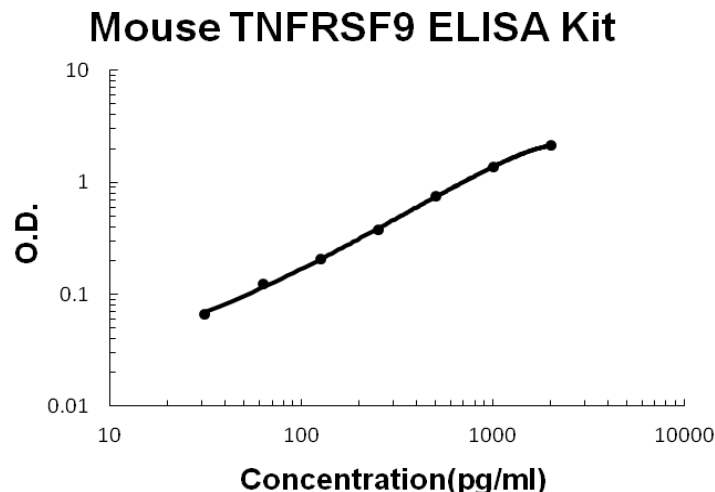
### Typical Data Obtained from Mouse TNFRSF9

(TMB reaction incubate at 37°C for 20 min)

|                      |       |       |       |       |       |       |       |       |
|----------------------|-------|-------|-------|-------|-------|-------|-------|-------|
| Concentration(pg/ml) | 0.0   | 31.2  | 62.5  | 125   | 250   | 500   | 1000  | 2000  |
| O.D                  | 0.039 | 0.066 | 0.122 | 0.206 | 0.372 | 0.748 | 1.375 | 2.121 |

### Typical Mouse TNFRSF9 ELISA Kit Standard Curve

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



|                         |   |
|-------------------------|---|
| <b>Range</b>            | 31.2pg/ml-2000pg/ml   |
| <b>Sensitivity</b>      | < 10pg/ml   |
| <b>Specificity</b>      | Natural and recombinant mouse TNFRSF9                       |
| <b>Cross-reactivity</b> | 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)        | 319                   | 746  | 1253 | 286                   | 833  | 1353 |
| Standard deviation | 12.76                 | 28.3 | 66.5 | 16.6                  | 37.5 | 94.7 |
| CV(%)              | 4                     | 3.8  | 5.3  | 5.8                   | 4.5  | 7    |

## Principle

OriGene's mouse TNFRSF9 ELISA Kit was based on standard sandwich enzyme-linked immune-sorbent assay technology. A monoclonal antibody from rat specific for TNFRSF9 has been precoated onto 96-well plates. Standards(NSO, V24-L187) and test samples are added to the wells, a biotinylated detection polyclonal antibody from goat specific for TNFRSF9 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 mouse TNFRSF9 amount of sample captured in plate.

## Kit Components

| Description   | Quantity              |
|---|-----------------------|
| 96-well plate precoated with anti- mouse TNFRSF9 antibody | 1                     |
| Lyophilized recombinant mouse TNFRSF9 standard            | 10ng/tubex2           |
| Biotinylated anti- mouse TNFRSF9 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                  |

## Material 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 H<sub>2</sub>O 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 Na<sub>2</sub>HPO<sub>4</sub> and 0.2g NaH<sub>2</sub>PO<sub>4</sub> to 1000ml distilled water and adjust pH to 7.2-7.6. Finally, adjust the total volume to 1L.

## **Notice for Application of Kit**

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.

## **Preparation**

### **1. 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.

### **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 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 (20-200ng/ml).** The working dilution is 1:100. i.e. Add 1µl sample into 99 µl sample diluent buffer.
- **Medium target protein concentration (2-20ng/ml).** The working dilution is 1:10. i.e. Add 10µl sample into 90 µl sample diluent buffer.
- **Low target protein concentration (31.2-2000pg/ml).** The working dilution is 1:2. i.e. Add 50µl sample to 50 µl sample diluent buffer.
- **Very Low target protein concentration ≤ 31.2pg/ml).** No dilution necessary, or the working dilution is 1:2.

### **3. Reagent Preparation and Storage**

- A. Reconstitution of the mouse TNFRSF9 standard: TNFRSF9 standard solution should be prepared no more than 2 hours prior to the experiment. Two tubes of TNFRSF9 standard (10ng per tube) are included in each kit. Use one tube for each experiment.
  - a. 10,000pg/ml of mouse TNFRSF9 standard solution: Add 1ml sample diluent buffer into one tube, keep the tube at room temperature for 10 min and mix thoroughly.
  - b. 2000pg/ml of mouse TNFRSF9 standard solution: Add 0.2ml of the above 10ng/ml TNFRSF9 standard solution into 0.8 ml sample diluent buffer and mix thoroughly.

- c. 1000pg/ml→31.2pg/ml of mouse TNFRSF9 standard solutions: Label 6 Eppendorf tubes with 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.2pg/ml respectively. Aliquot 0.3ml of the sample diluent buffer into each tube. Add 0.3ml of the above 2000pg/ml TNFRSF9 standard solution into 1st tube and mix. Transfer 0.3ml 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-mouse TNFRSF9 antibody working solution: The solution should be prepared no more than 2 hours prior to the experiment.
  - a. The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2ml more than total volume)
  - b. Biotinylated anti-mouse TNFRSF9 antibody should be diluted in 1:100 with the antibody diluent buffer and mixed thoroughly. (i.e. Add 1µl Biotinylated anti-mouse TNFRSF9 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.
  - a. The total volume should be: 0.1ml/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 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 TNFRSF9 detection curve should be prepared for each experiment. The user will decide sample dilution fold by crude estimation of TNFRSF9 amount in samples.

1. Aliquot 0.1ml per well of the 2000pg/ml, 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.2pg/ml mouse TNFRSF9 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 mouse cell culture supernates, serum or plasma (heparin, EDTA) to each empty well. **See "Sample Dilution Guideline" above for details.** It is recommended that each mouse TNFRSF9 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-mouse TNFRSF9 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 20-25 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 mouse TNFRSF9 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.<sub>450</sub>) = (the O.D.<sub>450</sub> of each well) – (the O.D.<sub>450</sub> of Zero well). The standard curve can be plotted as the relative O.D.<sub>450</sub> of each standard solution (Y) vs. the respective concentration of the standard solution (X). The mouse TNFRSF9 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.

### **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 20-25 min.
5. Add TMB stop solution and read.

### **Background**

CD137 is a member of the tumor necrosis factor (TNF) receptor family. Its alternative names are tumor necrosis factor receptor superfamily member 9 (TNFRSF9), 4-1BB and induced by lymphocyte activation (ILA). CD137 gene was localized to chromosome 1p36. Members of this receptor family and their structurally related ligands are important regulators of a wide variety of physiological processes and play an especially important role in the regulation of immune responses. CD137 can be expressed by activated T cells, but to a larger extent on CD8 than on CD4 T cells. The best characterized activity of CD137 is its costimulatory activity for activated T cells. Crosslinking of CD137 enhances T cell proliferation, IL-2 secretion survival and cytolytic activity. Further, it can enhance immune activity to eliminate tumors in mice.

### **Reference**

1. Jang, I K; Lee Z H, Kim Y J, Kim S H, Kwon B S (Jan. 1998). "Human 4-1BB (CD137) signals are mediated by TRAF2 and activate nuclear factor-kappa B". *Biochem. Biophys. Res. Commun. (UNITED STATES)* 242 (3): 613–20.
2. Arch, R H; Thompson C B (Jan. 1998). "4-1BB and Ox40 are members of a tumor necrosis factor (TNF)-nerve growth factor receptor subfamily that bind TNF receptor-associated factors and activate nuclear factor kappaB". *Mol. Cell. Biol. (UNITED STATES)* 18 (1): 558–65. ISSN 0270-7306. PMC 121523. PMID 9418902
3. Schwarz, H., Arden, K., Lotz, M. CD137, a member of the tumor necrosis factor receptor family, is located on chromosome 1p36, in a cluster of related genes, and colocalizes with several malignancies. *Biochem. Biophys. Res. Commun.* 235: 699-703, 1997.