



TNFRSF11A (Human) ELISA Kit

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96 assays

Version: 06

Intended for research use only

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Table of Contents

Introduction	3
Intended Use	3
Background	3
Principle of the Assay	3
General Information	4
Materials Supplied	4
Storage Instruction	4
Materials Required but Not Supplied	4
Precautions for Use	5
Assay Protocol	6
Reagent Preparation	6
Sample Preparation	7
Assay Procedure	7
Data Analysis.....	9
Calculation of Results.....	9
Performance Characteristics	9
Resources.....	10
References	10
Plate Layout	11

Introduction

Intended Use

Sandwich ELISA kit for quantitative detection of human RANK in cell culture supernates and serum.

Background

Receptor Activator of Nuclear Factor κ B (RANK), also known as TRANCE Receptor, is a type I membrane protein that is expressed on the surface of osteoclasts and is involved in their activation upon ligand binding. RANK is a recently described TNF receptor family member, and its ligand, RANKL, promote survival of dendritic cells and differentiation of osteoclasts. RANK contains 383 amino acids in its intracellular domain (residues 234-616), which contain three putative TRAF-binding domains (termed I, II, and III). RANK interacts with various TRAFs through distinct motifs and activates NF-kappaB via a novel TRAF6 interaction motif, which then activates NIK, thus leading to NF-kappaB activation, whereas RANK most likely activates JNK through a TRAF2-interacting region in RANK. The standard in this kit is recombinant human RANK with the sequence of Q29-G213 aa. It is a dipolymer which compose of two chains, and the molecular weight of each is 48 kda.

Principle of the Assay

TNFRSF11A (Human) ELISA Kit was based on standard sandwich enzyme-linked immune-sorbent assay technology. A monoclonal antibody from mouse specific for RANK has been precoated onto 96-well plates. Standards (Expression system for standard: NSO, Immunogen sequence: Q29-G213) and test samples are added to the wells, a biotinylated detection polyclonal antibody from goat specific for RANK 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 RANK amount of sample captured in plate.

General Information

Materials Supplied

List of component

Component	Amount
96-well plate precoated with anti- human RANK antibody	96 (8x12) wells
Lyophilized recombinant human RANK standard	10 ng/tubex2
Biotinylated anti- human RANK antibody, dilution 1:100	130 μ L
Avidin-Biotin-Peroxidase Complex (ABC), dilution 1:100	130 μ L
Sample diluent buffer	30 mL
Antibody diluent buffer	12 mL
ABC diluent buffer	12 mL
TMB color developing agent	10 mL
TMB stop solution	10 mL
Adhesive cover	4 slides

Storage Instruction

Store at 4°C for 6 months, at -20°C for 12 months. Avoid multiple freeze-thaw cycles.

Materials Required but Not Supplied

- ✓ Microplate reader in standard size.
- ✓ Automated plate washer.
- ✓ Adjustable pipettes and pipette tips. Multichannel pipettes are recommended in the condition of large amount of samples in the detection.
- ✓ Clean tubes and Eppendorf tubes.
- ✓ Washing buffer (neutral PBS or TBS).
 - Preparation of 0.01 M TBS:
Add 1.2 g Tris, 8.5 g NaCl; 450 μ L of purified acetic acid or 700 μ L of concentrated hydrochloric acid to 1000 mL H₂O and adjust pH to 7.2-7.6. Finally, adjust the total volume to 1 L.
 - Preparation of 0.01 M PBS:
Add 8.5 g sodium chloride, 1.4 g Na₂HPO₄ and 0.2 g NaH₂PO₄ to 1000 mL distilled water and adjust pH to 7.2-7.6. Finally, adjust the total volume to 1 L.

Precautions for Use

Please read the following instructions before starting the experiment.

- ✓ 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.
- ✓ The TMB Color Developing agent is colorless and transparent before using, contact us freely if it is not the case.
- ✓ Before using the Kit, spin tubes and bring down all components to the bottom of tubes.
- ✓ Duplicate well assay is recommended for both standard and sample testing.
- ✓ Don't let 96-well plate dry, for dry plate will inactivate active components on plate.
- ✓ Don't reuse tips and tubes to avoid cross contamination.
- ✓ Avoid using the reagents from different batches together.
- ✓ 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.

Assay Protocol

Reagent Preparation

- Reconstitution of the human RANK standard: RANK standard solution should be prepared no more than 2 hours prior to the experiment. Two tubes of RANK standard (10 ng per tube) are included in each kit. Use one tube for each experiment.
 - ✓ 10,000 pg/mL of human RANK standard solution: Add 1 mL sample diluent buffer into one tube, keep the tube at room temperature for 10 min and mix thoroughly.
 - ✓ 4000 pg/mL of human RANK standard solution: Add 0.4 mL of the above 10 ng/mL RANK standard solution into 0.6 mL sample diluent buffer and mix thoroughly.
 - ✓ 2000 pg/mL→62.5 pg/mL of human RANK standard solutions: Label 6 Eppendorf tubes with 2000 pg/mL, 1000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, respectively. Aliquot 0.3 mL of the sample diluent buffer into each tube. Add 0.3 mL of the above 4000 pg/mL RANK standard solution into 1st tube and mix. Transfer 0.3 mL from 1st tube to 2nd tube and mix. Transfer 0.3 mL from 2nd tube to 3rd tube and mix, and so on.

Note: The standard solutions are best used within 2 hours. The 10 ng/mL standard solution may be stored at 4°C for up to 12 hours, or at -20°C for up to 48 hours. Avoid repeated freeze-thaw cycles.
- Preparation of biotinylated anti-human RANK antibody working solution: The solution should be prepared no more than 2 hours prior to the experiment.
 - ✓ The total volume should be: 0.1 mL/well x (the number of wells). (Allowing 0.1-0.2 mL more than total volume)
 - ✓ Biotinylated anti-human RANK antibody should be diluted in 1:100 with the antibody diluent buffer and mixed thoroughly. (i.e. Add 1 µL Biotinylated anti- human RANK antibody to 99 µL antibody diluent buffer.)
- 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: 0.1 mL/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 1 µL ABC to 99 µL ABC diluent buffer.)

Sample Preparation

✓ 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 supernate: Remove particulates by centrifugation, analyze immediately or aliquot and store 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 10 min. Analyze the serum immediately or aliquot and store frozen 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 (40-400 ng/mL). The working dilution is 1:100. i.e. Add 3 µL sample into 297 µL sample diluent buffer.
- Medium target protein concentration (4-40 ng/mL). The working dilution is 1:10. i.e. Add 25 µL sample into 225 µL sample diluent buffer.
- Low target protein concentration (62.5-4000 pg/mL). The working dilution is 1:2. i.e. Add 100 µL sample to 100 µL sample diluent buffer.
- Very Low target protein concentration (0-62.5 pg/mL). No dilution necessary, or the working dilution is 1:2.

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 RANK detection curve should be prepared for each experiment. The user will decide sample dilution fold by crude estimation of RANK amount in samples.

1. Aliquot 0.1 mL per well of the 4000 pg/mL, 2000 pg/mL, 1000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL human RANK standard solutions into the precoated 96-well plate. Add 0.1 mL of the sample diluent buffer into the control well (Zero well). Add 0.1 mL of each properly diluted sample of human cell culture supernates or serum to each empty well. See “Sample Dilution Guideline” above for details. It is recommend that each human RANK standard solution and each sample be measured in duplicate.

2. Seal the plate with the a new adhesive cover provided 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 0.1 mL of biotinylated anti-human RANK antibody working solution into each well, seal the plate with a new adhesive cover provided and incubate at 37°C for 60 min.
5. Wash the plate 3 times with 0.01 M TBS or 0.01 M 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 0.1 mL of prepared ABC working solution into each well, seal the plate with a new adhesive cover provided and incubate at 37°C for 30 min.
7. Wash plate 5 times with 0.01 M TBS or 0.01 M 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 90 µL of prepared TMB color developing agent into each well, seal the plate with a new adhesive cover provided and incubate at 37°C in dark for 15-20 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 RANK standard solutions; the other wells show no obvious color.*)
9. Add 0.1 mL of prepared TMB stop solution into each well. The color changes into yellow immediately.
10. Read the O.D. absorbance at 450 nm in a microplate reader within 30 min after adding the stop solution.

✓ 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 0.01 M TBS.
3. Add ABC working solution and incubate the plate at 37°C for 30 min. Wash plate 5 times with 0.01 M TBS.
4. Add TMB color developing agent and incubate the plate at 37°C in dark for 15-20 min.
5. Add TMB stop solution and read.

Data Analysis

Calculation of Results

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 RANK 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 RANK

Concentration (pg/mL)	0	62.5	125	250	500	1000	2000	4000
O.D.	0.018	0.196	0.335	0.557	0.914	1.488	1.939	2.148

(TMB reaction incubate at 37°C for 15-20 min)

Performance Characteristics

- Range: 62.5 - 4000 pg/mL
- Sensitivity: < 2 pg/mL
- Specificity: Natural and recombinant human RANK
- Cross-reactivity: There is no detectable cross-reactivity with other relevant proteins.
- 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)	655	1863	2533	639	1957	2668
Standard deviation	26.2	68.93	136.8	37.1	90	176.1
CV (%)	4	3.7	5.4	5.8	4.6	6.6

Resources

References

1. Darnay B G; Haridas V, Ni J, Moore P A, Aggarwal B B (Aug. 1998). "Characterization of the intracellular domain of receptor activator of NF-kappaB (RANK). Interaction with tumor necrosis factor receptor-associated factors and activation of NF-kappab and c-Jun N-terminal kinase". J. Biol. Chem. (UNITED STATES) 273 (32): 20551-5.
2. Darnay, B G; Ni J, Moore P A, Aggarwal B B (Mar. 1999). "Activation of NF-kappaB by RANK requires tumor necrosis factor receptor-associated factor (TRAF) 6 and NF-kappaB-inducing kinase. Identificatoin of a novel TRAF6 interaction motif". J. Biol. Chem. (UNITED STATES) 274 (12): 7724-31.

Plate Layout

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