

## Mouse Receptor Activator of NF- $\kappa$ B (RANK) ELISA Kit (mRANK-ELISA)

*Cat. No. EK0830*

*96 Tests in 8 x 12 divisible strips*

### Background

Receptor Activator of Nuclear Factor- $\kappa$ 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, promotes 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). Darnay BG et al. demonstrated that RANK interacts with various TRAFs through distinct motifs and activates NF- $\kappa$ B via a novel TRAF6 interaction motif, which then activates NIK, thus leading to NF- $\kappa$ B activation, whereas RANK most likely activates JNK through a TRAF2-interacting region in RANK.

ScienCell's mouse RANK ELISA kit is based on standard sandwich enzyme-linked immune-sorbent assay technology. Mouse RANK-specific polyclonal antibodies are pre-coated onto 8 x 12 divisible strips. The mouse-specific detection polyclonal antibodies are biotinylated. The test samples and biotinylated detection antibodies are subsequently added to the wells and then washed with PBS or TBS buffer. Avidin-Biotin-Peroxidase Complex is added and unbound conjugates are washed away with PBS or TBS buffer. HRP substrate TMB is used to visualize HRP enzymatic reaction. TMB is catalyzed by HRP to produce a blue color product that changes to yellow after adding acidic stop solution. The intensity of yellow is proportional to the amount of mouse RANK in the sample that is captured on the strips.

<b>Size</b>	96 Tests in 8 x 12 divisible strips
<b>Assay type</b>	Sandwich ELISA
<b>Range</b>	62.5 pg/ml- 4000 pg/ml
<b>Sensitivity</b>	< 2 pg/ml
<b>Specificity</b>	No detectable cross-reactivity with any other cytokine.
<b>Storage</b>	Store at 4°C for frequent use, at -20°C for infrequent use. Avoid multiple freeze-thaw cycles.
<b>Shipping</b>	Shipped with gel ice.
<b>Expiration</b>	Four months at 4°C and eight months at -20°C.

<b>Application</b>	For quantitative detection of mouse RANK in tissue lysates or cell culture supernatants. The significance of detection in serum, plasma is undetermined.
<b>Kit components</b>	<ol style="list-style-type: none"> <li>1. Lyophilized recombinant mouse RANK standard: 10 ng/tube×2.</li> <li>2. 8 x 12 divisible strips pre-coated with anti- mouse RANK antibody.</li> <li>3. Sample diluent buffer: 30 ml</li> <li>4. Biotinylated anti-mouse RANK antibody: 130µl, dilution 1:100.</li> <li>5. Antibody diluent buffer: 12ml.</li> <li>6. Avidin-Biotin-Peroxidase Complex (ABC): 130µl, dilution 1:100.</li> <li>7. ABC diluent buffer: 12 ml</li> <li>8. TMB color developing agent: 10 ml.</li> <li>9. TMB stop solution: 10 ml.</li> </ol>
<b>Materials</b>	1. Microplate reader.
<b>Required But</b>	2. Automated plate washer.
<b>Not Provided</b>	<ol style="list-style-type: none"> <li>3. Adjustable pipettes and pipette tips. Multichannel pipettes are recommended for large amount of samples.</li> <li>4. Clean tubes and Eppendorf tubes.</li> <li>5. Washing buffer (neutral PBS or TBS).</li> </ol> <p>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.</p> <p>Preparation of 0.01 M PBS: Add 8.5g NaCl, 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.</p>
<b>Usage</b>	This product is for research use only. It is not approved for use in humans, animals, or <i>in vitro</i> diagnostic procedures.

## Reference

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. Identification of a novel TRAF6 interaction motif". J. Biol. Chem. (UNITED STATES) 274 (12): 7724–31.

## Protocol for Mouse RANK ELISA (96-well format)

### Notes before you begin

1. To inspect the validity of experiment operation and the appropriateness of sample dilution proportion, a pilot experiment using standards and a small number of samples is recommended.
2. The TMB Color developing agent should be colorless and transparent before using.
3. Before using the kit, spin tubes and bring down all components to the bottom of tubes.
4. A duplicate well assay is recommended for both standard and samples.
5. Do not let wells dry, as this will inactivate active components in wells.
6. Do not reuse tips and tubes to avoid cross contamination.

7. Avoid using reagents from different batches.
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 be pre-warmed in 37°C for 30 minutes before use.

## **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 supernatants or tissue lysate:** 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 10 minutes. Analyze the serum immediately or aliquot and store frozen at -20°C.
- **Plasma:** Use heparin as an anticoagulant. Centrifuge for 10 minutes at 2000 x g within 30 minutes of collection. Analyze 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 mixed well with the diluent 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 ( $\leq 62.5$  pg/ml).** No dilution necessary, or the working dilution is 1:2.

### **Reagent Preparation and Storage**

A. Reconstitution of the mouse 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 mouse RANK standard solution: Add 1 ml sample diluent buffer into one tube, keep the tube at room temperature for 10 minutes and mix thoroughly.
- 4000 pg/ml of mouse RANK standard solution: Add 0.4ml of the above 10ng/ml RANK standard solution into 0.6ml sample diluent buffer and mix thoroughly.
- 2000 pg/ml→62.5 pg/ml of mouse 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 0.3ml of the sample diluent buffer into each tube. Add 0.3ml 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 0.3ml 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 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 RANK antibody working solution: The solution should be prepared no more than 2 hours prior to the experiment.

- The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume).
- Biotinylated anti-mouse RANK antibody should be diluted in 1:100 with the antibody diluent buffer and mixed thoroughly.

C. 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: 0.1ml/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.

### **Assay Procedure**

The ABC working solution and TMB color developing agent must be kept warm at 37°C for 30 minutes 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 0.1ml per well of the 4000pg/ml, 2000pg/ml, 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml mouse RANK standard solutions into the pre-coated 8 x 12 divisible strips. Add 0.1ml of the sample diluent buffer into the control well (**blank well**). Add 0.1ml of each properly diluted sample of mouse serum, plasma, tissue lysates or cell culture supernatants to each empty well. See “**Sample Dilution Guideline**” above for details. We recommend that each mouse RANK standard solution and each sample is measured in duplicate.
2. Seal the strips with the cover and incubate at 37°C for 90 minutes.
3. Remove the cover, discard plate content, and blot the strips 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 RANK antibody working solution into each well and incubate the strips at 37°C for 60 minutes.
5. Wash the strips 3 times with 0.01M TBS or 0.01M PBS, and each time let washing buffer stay in the wells for 1 minute. Discard the washing buffer and blot the strips onto paper towels or other absorbent material. (**Strips Washing Method:** Discard the solution in the wells without touching the side walls. Blot the strips 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 strips onto paper towels or other absorbent material).
6. Add 0.1ml of prepared ABC working solution into each well and incubate the strips at 37°C for 30 minutes.
7. Wash the strips 5 times with 0.01M TBS or 0.01M PBS, and each time let washing buffer stay in the wells for 1-2 minutes. Discard the washing buffer and blot the strips onto paper towels or other absorbent material. (See Step 5 for strip washing method).
8. Add 90 µl of prepared TMB color developing agent into each well and incubate the strips at 37°C in dark for 25-30 minutes (**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 RANK standard solutions; the other wells show no obvious color).
9. Add 0.1ml of prepared TMB stop solution into each well. The color changes to yellow immediately.
10. Read the O.D. absorbance at 450 nm in a microplate reader within 30 minutes after adding the stop solution.

For calculation, (the relative O.D.450) = (the O.D.450 of each well) – (the O.D.450 of blank 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 mouse 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.

### Summary

1. Add samples and standards and incubate the strips at 37°C for 90 minutes. Do not wash.
2. Add biotinylated antibodies and incubate the strips at 37°C for 60 minutes. Wash strips 3 times with 0.01M TBS.
3. Add ABC working solution and incubate the strips at 37°C for 30 minutes. Wash strips 5 times with 0.01M TBS.
4. Add TMB color developing agent and incubate the strips at 37°C in dark for 25-30 minutes.
5. Add TMB stop solution and read.

### **Typical Data Obtained from Mouse RANK**

(TMB reaction incubate at 37°C for 23 minutes)

Concentration (pg/ml)	0.0	62.5	125	250	500	1000	2000	4000
Absorbance (450 nm)	0.007	0.135	0.254	0.476	0.806	1.313	1.818	2.166

### **Typical Mouse RANK ELISA Kit Standard Curve**

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

