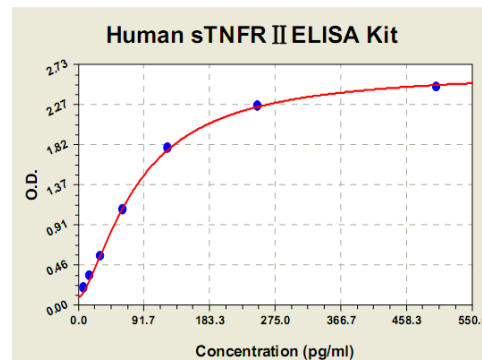


## KIT COMPONENTS

Component	Amount
96-well plate precoated with anti-human TNFRII antibody	1 Plate
Protein Standard: Lyophilized recombinant human TNFRII	2 tubes, 10 ng/tube
Sample Diluent Buffer	30 ml
Biotinylated Antibody (Anti-human TNFRII)	130 µl (100x)
Antibody Diluent Buffer	12ml
Avidin-Biotin-Peroxidase Complex (ABC) Solution	130 µl (100x)
ABC Diluent Buffer	12 ml
Tetramethylbenzidine (TMB) Color Developing Agent	10 ml
TMB Stop Solution	10 ml

### Storage

Store at 4°C. Cell Applications, Inc. recommends using the kit within 6 months of order.



X	pg/ml	0.0	7.8	15.6	31.3	62.5	125	250	500
Y	O.D.	0.450	0.049	0.214	0.339	0.566	1.095	1.790	2.482

**Figure 1: TNFRII Standard Curve.** Using the human TNFRII ELISA Kit, O.D. data was graphed against TNFRII protein concentration. The TMB reaction was incubated at 37° C for 10 min.

## BACKGROUND

Tumor necrosis factor-alpha (TNF-alpha), a proinflammatory cytokine, exerts its biological activity by signaling via its two receptors, TNFRI or p55, also classified as CD120a, and TNFRII, also referred to as p75 or CD120b. These two receptors are co-expressed in almost every cell in the body, but in contrast to the constitutive expression of TNFRI, the expression of TNFRII is strongly modulated by various cytokines including TNF-alpha, IL-1, IFN-gamma and other inflammatory stimuli like LPS. In addition, the extracellular parts of the receptors are proteolytically shed to the soluble forms by a matrix metalloproteinase-like enzyme (sTNFRI and sTNFRII). The soluble TNF receptors can neutralize TNF-alpha activities. Circulating levels of both sTNFRI and sTNFRII are elevated in patients with various diseases.<sup>1</sup> TNFRI is generally regarded as the dominant receptor in TNF-alpha biology; it is the high affinity receptor for soluble TNF-alpha (s TNF-alpha), and mediates many of the pro-inflammatory effects of this cytokine. However, TNF effects on T cells seem to be TNFRII-mediated.<sup>2</sup> The absence of TNFRII significantly reduces the dermatotoxic and lethal effects of TNF-alpha. TNFRII expression is also increased on microvascular endothelial cells (ECs) in the lung during adult respiratory distress syndrome (ARDS) and is associated with the enhanced expression of cell adhesion molecules including ICAM-1, VCAM-1 and of CD14 in this context. Following oligomerization by TNF-alpha, the receptors TNFRI and TNFRII associate with adapter molecules via specific protein-protein interactions. The subsequent recruitment of downstream molecules to the receptor complex enables propagation of the TNF-alpha signal. Two cellular responses to TNF-alpha have been well documented, the induction of cell death and the activation of gene transcription for cell survival. It was believed that TNF-induced apoptosis is mediated exclusively by TNFRI because TNFRII lacks death domain. However, it has been demonstrated that TNFRII enhances TNFRI-mediated apoptosis. Two transcription factors activated by TNF-alpha are nuclear factor kappaB (NFkappaB) and activating protein 1 (AP-1). Pathways that promote the activation of these transcription factors involve signaling molecules such as kinases, phospholipases, and sphingomyelinases. In addition to increased survival (anti-apoptotic) gene expression, NFkappaB and AP-1 also induce the expression of genes involved in inflammation, cell growth, and signal regulation.<sup>3</sup>

## Reference

- Nozaki, N. et al: J. Mol. Cell Cardiol. 30:2003-12, 1998
- Aspalter, R.M. et al: J. Leuk. Biol. 74:572-82, 2003
- Leong, K.G. & Karsan, A.: Histol Histopathol. 15:1303-25, 2000

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## ELISA OVERVIEW

Cell Applications ELISA Kits are based on standard sandwich enzyme-linked immunosorbent assay technology. Freshly prepared standards, samples, and solutions are recommended for best results.

1. Prepare test samples.
2. Prepare a protein standard of the target protein.
3. Add test samples and standards to the pre-coated 96-well plate. Do not wash.
4. Add biotinylated detection antibodies. Wash.
5. Add Avidin-Biotin-Peroxidase Complex (ABC) Solution. Wash.
6. Add Tetramethylbenzidine (TMB) Color Developing Agent, containing HRP substrate.
7. Add TMB Stop Solution
8. Subject the plate to analysis.

## NOTES:

- Before using the kit, quick spin tubes to bring down all solution to the bottom of tube.
- Duplicate assay wells are recommended for both standard and sample testing.
- Do not let the 96-well plate dry, this will lead to inactivation of plate components.
- When diluting samples and reagents, ensure that they are mixed completely and evenly.
- Pre-warm diluted ABC and TMB solutions at 37°C for 30 min before use to avoid variable temperature effects.
- For washes, use TBS or PBS. Do not touch well walls.
- A protein standard is included in the kit. A protein standard detection curve should be generated with each experiment, no more than 2 hours prior to the experiment.
- The user will determine sample dilution fold by estimation of target protein amount in samples.



## ELISA PROTOCOL

### Preparation of Test Samples

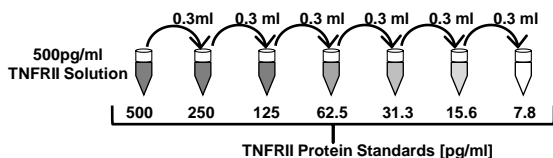
- Process Test Samples in the following manner:
  - Cell culture supernate, tissue lysate or body fluids:** Remove particulates by centrifugation, analyze immediately or aliquot and store at -20° C.
  - Serum:** Allow the serum to clot in a serum separator tube (about 30 min) at room temperature. Centrifuge at approximately 1000 X g for 15 min. Analyze the serum immediately or aliquot and store frozen at -20° C.
  - Plasma:** Collect plasma using heparin/EDTA/citrate as an anticoagulant. Centrifuge for 15 min at 1000 x g within 30 min of collection. Analyze immediately or aliquot and store frozen at -20° C.
- Estimate the concentration of the target protein in the sample and select a proper dilution factor such that the diluted target protein concentration falls within the 7.8-500 pg/ml standard curve range. Depending on the sample, several trial dilutions may be necessary. Dilute the sample using the provided diluent buffer, mixing well. Suggested working dilutions of samples are as follows:

Target Protein Concentration Range	Sample Working Dilution	Sample Vol.	Diluent Buffer Vol.
5-50 ng/ml	1:100	1 µl	99 µl
500-5000 pg/ml	1:10	10 µl	90 µl
7.8-500 pg/ml	1:2	50 µl	50 µl
≤7.8pg/ml	n/a	100µl	n/a

- If samples will be assayed within 24 hours, store at 2-8° C. For long-term storage, aliquot and freeze samples at -20° C. Avoid repeated freeze-thaw cycles.

### Preparation of Standard Solutions (7.8-500 pg/ml)

- Reconstitute the Lyophilized Recombinant Protein to make a 10,000 pg/ml human TNFR II solution. Add 1 ml Sample Diluent Buffer to a tube of lyophilized protein, keep the tube at room temperature for 10 min. Mix thoroughly.
- Add 0.05 ml of the mixed 10,000 pg/ml TNFR II solution to the eppendorf tube containing 0.95 ml diluent buffer and mix to make a 500 pg/ml TNFR II solution.
- Label 6 eppendorf tubes with the TNFR II protein concentrations to be prepared by serial dilution: 250pg/ml, 125pg/ml, 62.5pg/ml, 31.3pg/ml, 15.6pg/ml, 7.8pg/ml.
- Aliquot 0.3 ml of the Sample Diluent Buffer to the labeled tubes.
- Serially dilute the protein standards into their respectively labeled tubes. Transfer 0.3 ml from the 500pg/ml TNFR II Solution to the 250pg/ml eppendorf tube and mix thoroughly. Transfer 0.3 ml of the 250 pg/ml solution to the 125pg/ml tube and mix thoroughly, Transfer 0.3 ml of the 125pg/ml solution to the 62.5pg/ml tube and mix, and so on to make the 31.3, 15.6 and 7.8 pg/ml solutions.
- Store at 4° C until use.



### Loading the 96-well Plate

- Aliquot 0.1 ml of the sample diluent buffer into a control well to serve as the Blank. This will yield the O.D.450(Blank) reading.
- Aliquot 0.1 ml of the standard solutions of the **Preparation of Standard Solutions** (7.8-500pg/ml) into empty wells of the precoated 96-well plate. Duplicate measurements of standards are recommended.

- Aliquot 0.1 ml of each properly diluted test sample to empty wells prepared in **Step 2**. Duplicate measurements of each test sample are recommended.
- Cover the 96-well plate and incubate at 37° C for 90 min.
- During the **Step 13** incubation period, prepare a stock of Biotinylated 1:100 Antibody Working Solution. Count the number of reactions and multiply by 0.1 ml/well for the Working Solution total volume (preparation of 1-2 reactions in excess of the number of wells is recommended). Dilute the Biotinylated Antibody to 1:100 in Antibody Diluent Buffer and mix thoroughly. Use the working solution within 2 hours.
- Upon completion of the 90 min incubation of **Step 13**, remove the cover of the 96 well plate and discard plate well contents. Blot the plate onto paper towels or other absorbent material. DO NOT let the wells completely dry at any time.
- Add 0.1 ml of the Biotinylated 1:100 Antibody Working Solution (prepared in **Step 14**) to each well and incubate the plate at 37° C for 60 min.
- During the incubation period of **Step 16**, prepare a stock of ABC Working Solution. Count the number of reactions and multiply by 0.1 ml/well for the Working Solution total volume (preparation of 1-2 reactions in excess of the number of wells is recommended). Dilute the ABC Stock Solution to 1:100 in ABC Diluent Buffer and mix thoroughly. Pre-warm the ABC working solution at 37° C for 30 min before use. Use the working solution within 1 hour.
- Upon completion of the 60 min incubation of **Step 16**, wash the plate 3 times with 0.3 ml TBS or PBS. For each wash, leave washing buffer in the wells for 1-2 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material.
- Add 0.1 ml of prepared ABC Working Solution (prepared in **Step 17**) to each well and incubate the plate at 37° C for 30 min.
- During the incubation period of **Step 19**, pre-warm TMB Color Developing Agent at 37° C for 30 min before use.
- Upon completion of the 30 min incubation of **Step 19**, wash the plate 5 times with 0.3 ml TBS or PBS. For each wash, leave the washing buffer in the wells for 1-2 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material.
- Add 90 µl of the pre-warmed TMB Color Developing Agent into each well and incubate at 37° C for 10-15 min (shades of blue can be seen in the wells with the four most concentrated Protein Standard Solutions; the other control wells should show no obvious color).
- Add 0.1 ml of the TMB Stop Solution to each well. The acidic stop solution will change the mixture color to yellow. The yellow intensity is proportional to the amount of target protein captured by the plate.
- Read the O.D. absorbance at 450nm in a microplate reader within 30 min after adding the stop solution. These readings are the O.D.450(Reading).

### Calculating Protein Concentration

- For all wells, determine O.D.450(Relative):  

$$\text{O.D.450(Relative)} = \text{O.D.450(Reading)} - \text{O.D.450(Blank)}$$
- Plot the standard curve: plot O.D.450(Relative) of each standard solution (Y) vs. the respective concentration of the standard solution (X). See **Figure 1** for a typical standard curve.
- The TNFR II concentration of the samples can be interpolated from the standard curve. Multiply the interpolated concentration by the dilution factor to obtain the target protein concentration in the sample.

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