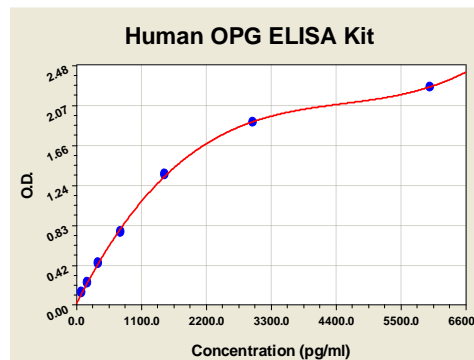


**KIT COMPONENTS**

Component	Amount
96-well plate pre-coated with anti-human OPG antibody	1 Plate
Protein Standard: Lyophilized recombinant human OPG	2 tubes, 10 ng/tube
Sample Diluent Buffer	30 ml
Biotinylated Antibody (Anti-human OPG)	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	93.8	187.5	375	750	1500	3000	6000	
Y	O.D.	0.450	0.020	0.134	0.239	0.438	0.759	1.363	1.892	2.259

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

**BACKGROUND**

Osteoprotegerin (OPG), a member of the tumor necrosis factor receptor superfamily, is a soluble decoy receptor for the osteoclast differentiation factor receptor-activator of nuclear factor B ligand (RANKL) that inhibits interaction between RANKL and its membrane-bound receptor RANK. OPG protein comprises 401 amino acid residues of which 21 form a signal peptide that is cleaved generating a mature form of 380 amino acid residues. At the N-terminus, there are four domains (D1-4), which have Cys-rich TNFR homologue motifs and are necessary and sufficient for binding to RANKL, and for inhibiting osteoclastic differentiation and activity. At the C-terminus, there are tandem death-domain homologue regions (D5 and D6) followed by a heparin-binding site (D7) and , at position 400, there is a Cys residue required for homodimerization of OPG. OPG is produced as a monomer (55-62 kDa), undergoes homodimerization, and is secreted as a disulfide-linked homodimeric glycoprotein with four or five glycosylation sites, generating a mature form of OPG of 110-120 kDa. The dimeric form of OPG exhibits a much higher affinity to RANKL.<sup>1</sup> The RANKL/OPG/RANK axis has been shown to regulate bone remodeling. RANKL is essential for osteoclast formation, function and survival and each of these activities are prevented by OPG. OPG has traditionally been linked to a number of bone-related diseases. However, there is additional evidence that OPG can promote cell survival by inhibiting TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. Moreover, OPG is involved in the vasculature.<sup>2</sup> In addition, it has been demonstrated that RANKL/OPG/RANK system is linked to the development of atherosclerosis and plaque destabilization. Circulating OPG levels are increased in patients with acute coronary syndrome, and enhanced expression has been found within symptomatic carotid plaques. There are also a few studies that show a relationship between OPG and cardiovascular disease (CVD) and related mortality in the general population.<sup>3</sup> Additionally the RANKL/OPG/RANK axis was found to be involved in central thermoregulation.<sup>4</sup>

**Reference**

1. D'Amelio, P. et al: J Endocrinol Invest. 32(4 Suppl):6-9, 2009
2. Kiechl, S. et al: Expert Rev Cardiovasc Ther. 4:901-11, 2006
3. Caidahl, K. et al: Arterioscler Thromb Vasc Biol. 30:1684-6, 2010
4. Hanada, R. et al: Nature 462: 505-9,2009

**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.

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## ELISA PROTOCOL

### Preparation of Test Samples

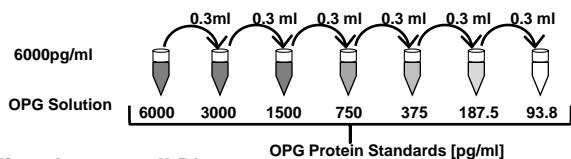
- Process Test Samples in the following manner:
  - Cell culture supernate, tissue lysate or body fluids:** Remove particulates by centrifugation. analyze immediately or aliquot and store at -20° C.
  - Serum:** Allow the serum to clot in a serum separator tube (about 30 min) at room temperature. Centrifuge at approximately 1000 x g for 15 min. Analyze the serum immediately or aliquot and store frozen at -20° C..
- 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 93.8-6000 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.
60-600 ng/ml	1:100	1 µl	99 µl
6-60 ng/ml	1:10	10 µl	90 µl
93.8-6000 pg/ml	1:2	50 µl	50 µl
≤93.8 pg/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 (93.8-6000 pg/ml)

- Reconstitute the Lyophilized Recombinant Protein to make a 10,000 pg/ml human OPG 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 0.6 ml of the mixed 10,000 pg/ml OPG solution to the eppendorf tube containing 0.4 ml diluent buffer and mix to make a 6000 pg/ml OPG solution.
- Label 6 eppendorf tubes with the human OPG protein concentrations to be prepared by serial dilution :3000pg/ml, 1500pg/ml, 750pg/ml, 375pg/ml, 187.5pg/ml, 93.8pg/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 6000pg/ml OPG Solution to the 3000pg/ml eppendorf tube and mix thoroughly. Transfer 0.3 ml of the 3000 pg/ml solution to the 1500pg/ml tube and mix thoroughly, Transfer 0.3 ml of the 1500pg/ml solution to the 750pg/ml tube and mix, and so on to make the 375, 187.5 and 93.8 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** (93.8-6000pg/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.
- Add 90 µl of the pre-warmed TMB Color Developing Agent into each well and incubate at 37° C for 8-12 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).
- 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 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 human OPG 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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