CELL APPLICATIONS, INC.

Human OPN ELISA Kit

Cat. No. CL0482
96-wells

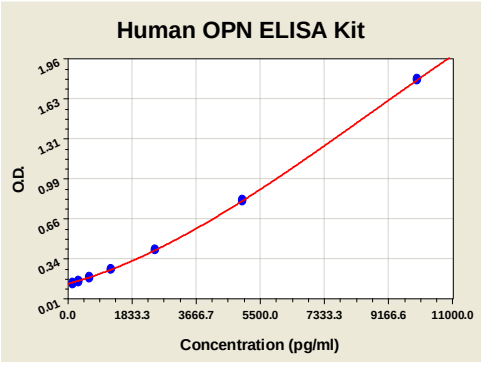
Target Protein Species:	Human
Range	156pg/ml – 10000pg/ml
Specificity	No detectable cross-reactivity with any other cytokine

KIT COMPONENTS

Component	Amount
96-well plate precoated with anti-human OPN antibody	1 Plate
Protein Standard: Lyophilized recombinant human OPN	2 tubes, 100 ng/tube
Sample Diluent Buffer	30 ml
Biotinylated Antibody (Anti-human OPN)	130 µl (100x)
Antibody Diluent Buffer	12ml
Avidin-Biotin-Peroxidase Complex (ABC) Solution	130 µl (100x)
ABC Diluent Buffer	12 ml
Tetramethyllbenzidine (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	156	313	625	1250	2500	5000	10000
Y	O.D.450	0.139	0.147	0.162	0.191	0.256	0.409	0.812	1.792

Figure 1: OPN Standard Curve. Using the human OPN ELISA Kit, O.D. data was graphed against OPN protein concentration. The TMB reaction was incubated at 37° C for 23 min.

BACKGROUND

Osteopontin (OPN, also known as early T cell activation gene-1, or eta-1) is a secreted, multifunctional glycoprotein that exists either as a full-length molecule or as proteolytic fragments. Multiple forms of OPN have been described, including various glycosylated, phosphorylated, sulfated, and nonsulfated species, ranging from 25 to 75 kDa. OPN is synthesized by many cell types and involved in various physiologic and pathologic processes, including cell adhesion, migration, differentiation, apoptosis, angiogenesis, inflammatory responses, and tumor metastasis.[1] The different effects that OPN elicit are attributable to its multiple receptors, binding sites, and its various forms. One of the major serine proteases to cleave Opn is thrombin, giving rise to a 24-kDa and a 45-kDa fragment. The 45-kDa fragment has multiple functional advantages in processes such as cell attachment, migration, and spreading through binding to alpha9beta1 and alpha4beta1. OPN is bound by multiple integrins. In addition, there is evidence that CD44 can also bind OPN possibly via the v6 and v7 variants. Stem cells are known to express CD44 and alpha4 integrin, both of which are receptors capable of interacting with OPN.[2] OPN binds to cells, activating multiple and varied signaling pathways. It was reported that OPN upregulates alphavbeta3 integrin-mediated Janus kinase 2 (JAK2) phosphorylation and STAT3 activation in breast cancer.[3] OPN was initially identified in osteoblasts as a mineralization-modulatory matrix protein. Now OPN has been studied as a multifunctional protein that is upregulated in a variety of acute and chronic inflammatory conditions. In the context of atherosclerosis, OPN is generally regarded as a proinflammatory and proatherogenic molecule. However, the role of OPN in vascular calcification (VC), is that of a negative regulator because it is an inhibitor of calcification and an active inducer of decalcification. In addition to being produced by cells of osteoblastic lineage, OPN has been shown to play important roles in chemotaxis, adhesion, and proliferation, all of which allow it to mediate inflammation and immunity to infectious diseases. Additionally, OPN can function as a Th1 cell cytokine, enhancing IL-12 while inhibiting expression of the Th2 cell cytokine IL-10. Furthermore, OPN can alter the sensitivity of hematopoietic cells to other cytokine stimuli.[4]

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 Tetramethyllbenzidine (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.

Reference

1. Haylock, D.N. & Nilsson, S.K.: Br. J. Haematol. 134:467-74, 2006
2. Scatena, M. et al: Arterioscler Thromb Vasc Biol. 27:2302-9, 2007
3. Behera, R. et al: Carcinogen. 31:192-200, 2010
4. Cho, H.J. et al: Curr Atheroscler Rep. 11:206-13, 2009

ELISA PROTOCOL

Preparation of Test Samples

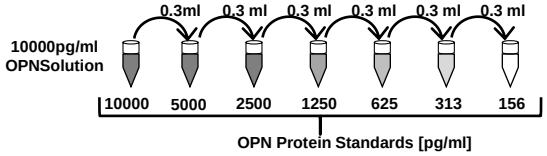
1. Process Test Samples in the following manner:
   - **Cell culture supernate, tissue lysate or body fluids**: Remove particulates by centrifugation.
   - **Serum**: Allow the serum to clot in a serum separator tube (about 30 min) at room temperature. Centrifuge at approximately 1000 x g for 15 min. 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. EDTA and citrate are not recommended as the anticoagulant.
   - 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 156-10000 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.
100-1000 ng/ml	1:100	1 µl	99 µl
10-100 ng/ml	1:10	10 µl	90 µl
156-10000 pg/ml	1:2	50 µl	50 µl
≤156 pg/ml	n/a	100µl	n/a

2. 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 (156-10000 pg/ml)

3. Reconstitute the Lypophilized Recombinant Protein to make a 10.000 pg/ml human OPN solution. Add 1 ml Sample Diluent Buffer to a tube of lypophilized protein, keep the tube at room temperature for 10 min. Mix thoroughly.
4. Label 6 eppendorf tubes with the OPN protein concentrations to be prepared by serial dilution: 5000pg/ml, 2500pg/ml, 1250pg/ml, 625pg/ml, 313pg/ml, 156pg/ml.
5. Aliquot 0.3 ml of the Sample Diluent Buffer to the labeled tubes.
6. Serially dilute the protein standards into their respectively labeled tubes. Transfer 0.3 ml from the 10000pg/ml OPN Solution to the 5000pg/ml eppendorf tube and mix thoroughly. Transfer 0.3 ml of the 5000 pg/ml solution to the 2500pg/ml tube and mix thoroughly, Transfer 0.3 ml of the 2500pg/ml solution to the 1250pg/ml tube and mix, and so on to make the 625, 313 and 156 pg/ml solutions.
7. Store at 4° C until use.



Loading the 96-well Plate

8. 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.
9. Aliquot 0.1 ml of the standard solutions of the **Preparation of Standard Solutions** (156-10000pg/ml) into empty wells of the precoated 96-well plate. Duplicate measurements of standards are recommended.
10. 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.
11. Cover the 96-well plate and incubate at 37° C for 90 min.
12. During the **Step 11** 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.
13. Upon completion of the 90 min incubation of **Step 11**, 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.
14. Add 0.1 ml of the Biotinylated 1:100 Antibody Working Solution (prepared in **Step 12**) to each well and incubate the plate at 37° C for 60 min.
15. During the incubation period of **Step 14**, 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.
16. Upon completion of the 60 min incubation of **Step 14**, 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.
17. Add 0.1 ml of prepared ABC Working Solution (prepared in **Step 15**) to each well and incubate the plate at 37° C for 30 min.
18. During the incubation period of **Step 17**, pre-warm TMB Color Developing Agent at 37° C for 30 min before use.
19. Upon completion of the 30 min incubation of **Step 17**, 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.
20. Add 90 µl of the pre-warmed TMB Color Developing Agent into each well and incubate at 37° C for 20-25 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).
21. 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.
22. 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):
  O.D.450(Relative) = O.D.450(Reading) – 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 OPN 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.