CELL APPLICATIONS, INC.

# Human E-selectin ELISA Kit

Cat. No. CL0501
96-wells

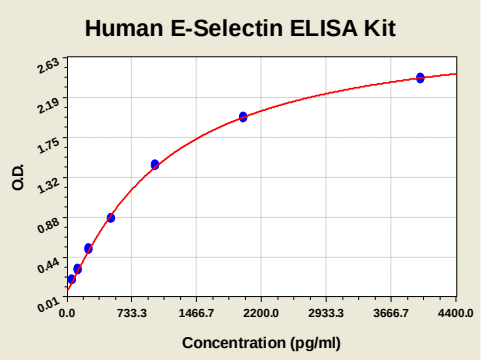
| | |
|---|---|
| **Target Protein Species:** | Human |
| **Range** | 62.5 pg/ml – 4000pg/ml |
| **Specificity** | No detectable cross-reactivity with IGF-1 |

## KIT COMPONENTS

| Component | Amount |
|---|---|
| 96-well plate precoated with anti-human E-selectin antibody | 1 Plate |
| Protein Standard: Lyophilized recombinant human E-selectin | 2 tubes, 10 ng/tube |
| Sample Diluent Buffer | 30 ml |
| Biotinylated Antibody (Anti-human E-selectin) | 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℃. Cell Applications, Inc. recommends using the kit within 6 months of order.




| X | pg/ml | 0.0 | 62.5 | 125 | 250 | 500 | 1000 | 2000 | 4000 |
|---|---|---|---|---|---|---|---|---|---|
| Y | O.D.450 | 0.053 | 0.191 | 0.306 | 0.531 | 0.867 | 1.446 | 1.977 | 2.394 |

**Figure 1:** E-selectin **Standard Curve.** Using the human E-selectin ELISA Kit, O.D. data was graphed against E-selectin protein concentration. The TMB reaction was incubated at 37° C for 20 min.

## BACKGROUND

E-selectin is an endothelial cell-specific membrane glycoprotein that mediates slow rolling and stable arrest of leukocytes on endothelium during inflammation. E-selectin binds two ligands, PSGL-1 and E-selectin ligand-1 (ESL-1).[1,2] Some glycolipids can support E-selectin dependent rolling in vitro. Its function in mediating leukocyte rolling is largely redundant with that of P-selectin. E-selectin operates "downstream" from P-selectin, more toward the firm adhesion step of the cascade. E-selectin is not constitutively expressed in endothelial cells, but is transcriptionally induced by NF-κB and AP-1 in response to inflammatory cytokines such as IL-1β and TNF-α. Consequently, elevated E-selectin expression was reported in many types of inflammatory diseases including diabetes, atherosclerosis, rheumatoid arthritis, and cancer. In addition to E-selectin-mediated inflammation, many studies have suggested a potential involvement of E-selectin in the attachment and transmigration of circulatory metastatic cancer cells through the endothelium.[3] In addition, there is evidence that E-selectin may play a role in angiogenesis. It was shown that antibodies directed against E-selectin inhibited the formation of capillary-like tubes in vitro. It was reported that soluble E-selectin, which lacks the transmembrane and cytoplasmic domains, induces angiogenesis in the rat cornea and stimulates chemotaxis and tube formation of human dermal microvascular endothelial cells (HDMEC) through Src- and phosphatidylinositol 3-kinase-mediated pathways. E-selectin is closely associated with essential events of angiogenesis including endothelial cell proliferation, migration, and neovascularization.[4]

### Reference


1. Rosen, S.D. et al: Ann. Rev. Immunol. 22:129-56, 2003
2. Varki, A. et al: Proc. Natl. Acad. Sci. USA 91:7390-7, 1994
3. Mann, A.P. et al: PloS ONE 5:e13050, 2010
4. Koch, A. E. et al: Nature 376:517-9, 2002


## 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℃ 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

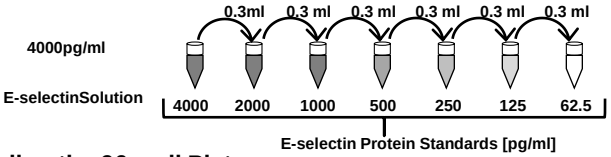
1. Process Test Samples in the following manner:
   - **Cell culture supernate, tissue lysate or body fluids**: Remove particulates by centrifugation.
   - **Serum**: Allow the serum to clot in a serum separator tube (about 2 hours) at room temperature. Centrifuge at approximately 1000 x g for 10 min. Analyze the serum immediately or aliquot and store frozen at -20° C.
   - **Plasma:** Collect plasma using heparin/EDTA/citrate as an anticoagulant. Centrifuge for 10 min at 1000 x g within 30 min of collection. Analyze immediately or aliquot and store frozen at -20° C. Heparin and citrate are not recommended as the anticoagulant..
2. 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 62.5-4000 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. |
|---|---|---|---|
| 40-400 ng/ml | 1:100 | 1 µl | 99 µl |
| 4-40 ng/ml | 1:10 | 10 µl | 90 µl |
| 62.5-4000 pg/ml | 1:2 | 50 µl | 50 µl |
| ≤62.5pg/ml | n/a | 100µl | n/a |

3. 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 (62.5-4000 pg/ml)

4. Reconstitute the Lypophilized Recombinant Protein to make a 10.000 pg/ml human E-selectin solution. Add 1 ml Sample Diluent Buffer to a tube of lypophilized protein, keep the tube at room temperature for 10 min. Mix thoroughly.
5. Add 0.4 ml of the mixed 10,000 pg/ml E-selectin solution to the eppendorf tube containing 0.6 ml diluent buffer and mix to make a 4000 pg/ml E-selectin solution.
6. Label 6 eppendorf tubes with the Human E-selectin protein concentrations to be prepared by serial dilution 2000pg/ml, : 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml,
7. Aliquot 0.3 ml of the Sample Diluent Buffer to the labeled tubes.
8. Serially dilute the protein standards into their respectively labeled tubes. Transfer 0.3 ml from the 4000pg/ml E-selectin Solution to the 2000pg/ml eppendorf tube and mix thoroughly. Transfer 0.3 ml of the 2000 pg/ml solution to the 1000pg/ml tube and mix thoroughly, Transfer 0.3 ml of the 1000 pg/ml solution to the 500pg/ml tube and mix, and so on to make the 250, 125 and 62.5 pg/ml solutions.
9. Store at 4° C until use.




### Loading the 96-well Plate

10. 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.
11. Aliquot 0.1 ml of the standard solutions of the **Preparation of Standard Solutions** (62.5-4000pg/ml) into empty wells of the precoated 96-well plate. Duplicate measurements of standards are recommended.
12. 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.
13. Cover the 96-well plate and incubate at 37° C for 90 min.
14. 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.
15. 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.
16. 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.
17. 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.
18. 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.
19. 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.
20. During the incubation period of **Step 19**, pre-warm TMB Color Developing Agent at 37° C for 30 min before use.
21. 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.
22. Add 90 µl of the pre-warmed TMB Color Developing Agent into each well and incubate at 37° C for 15-20 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).
23. 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.
24. 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 E-selectin 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.