CELL APPLICATIONS, INC.

mouse P-selectin ELISA Kit

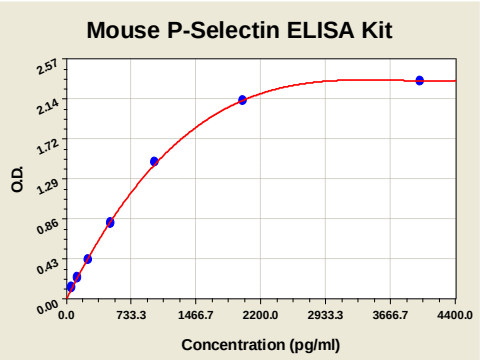
Cat. No. CL0506
96-wells

Target Protein Species: Mouse
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-mouse P-selectin antibody	1 Plate
Protein Standard: Lyophilized recombinant mouse P-selectin	2 tubes, 20 ng/tube
Sample Diluent Buffer	30 ml
Biotinylated Antibody (Anti-mouse P-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	0.015	0.131	0.234	0.423	0.820	1.471	2.125	2.340

Figure 1: P-selectin **Standard Curve.** Using the mouse P-selectin ELISA Kit, O.D. data was graphed against P-selectin protein concentration. The TMB reaction was incubated at 37° C for 14 min.

BACKGROUND

P-selectin (CD62P), the largest of the selectins, with a mass of 140kDa, extends approximately 40nm from the endothelial surface. Previous names include granule membrane protein 140 (GMP-140) and platelet activation dependent granule external membrane protein (PADGEM). It is a component of the membrane of the alpha and dense granules of platelets, and also of the membrane of the Weibel–Palade bodies of endothelial cells. In common with the other selectins, P-selectin has an N-terminal lectin domain, an epidermal growth factor motif, (generally) nine regulatory protein repeats, a transmembrane section and a short intracytoplasmic tail. Using P-selectin knockout mice, the importance of P-selectin-mediated cell adhesive interactions in the pathogeneses of inflammation, thrombosis, growth and metastasis of cancers has been clearly demonstrated.[1] On inflammatory and thrombogenic challenges, P-selectin rapidly translocates to the surface of these cells and contributes to the weak adhesion of leukocytes to stimulated endothelial cells and the heterotypic aggregation of activated platelets to leukocytes. A principal leukocyte ligand for P-selectin is P-selectin glycoprotein ligand 1 (PSGL-1), a disulfide bond-linked homodimer. It is expressed on a variety of human leukocytes, including neutrophils, monocytes, certain subsets of T lymphocytes, eosinophils, and basophils. PSGL-1 is the best-characterized selectin ligand that has been demonstrated to mediate leukocytes rolling on endothelium and leukocytes recruitment into inflamed tissue in vivo. In addition to its direct role in leukocyte capturing, PSGL-1 also functions as a signal-transducing receptor.[2] Moreover, cross-linking of PSGL-1 by P-selectin also primes leukocytes intracellularly for cytokine and chemoattractant-induced beta2-integrin activation for firm adhesion of leukocytes. Furthermore, P-selectin mediates heterotypic aggregation of activated platelets to cancer cells and adhesion of cancer cells to stimulated endothelial cells.[3] P-selectin, released from the cell surface, circulates as a soluble molecule in the plasma. It was shown that soluble P-selectin (sP-selectin) can exert procoagulant activity and it might therefore play an important role in thrombosis and acute coronary events. sP-selectin is often used as a marker of platelet activation.[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℃ 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. Frenette, P.S. & Wagner, D.D.: Thromb Haemost. 78:60-4, 1997
2. Moore, K.L. et al: J. Cell Biol. 128:661-71, 1995
3. Bird, M.I. et al: Biochem Soc Trans. 25:1199-206, 1997
4. Ridker, P.M. et al: Circulation 103:491-5, 2001

ELISA PROTOCOL

Preparation of Test Samples

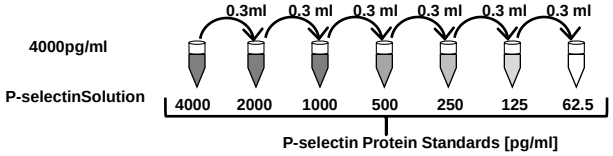
1. Process Test Samples in the following manner:
   - **Cell culture supernate, tissue lysate or body fluids**: Remove particulates by centrifugation.
   - **Serum**: Allow the serum to clot in a serum separator tube (about 2 hours) at room temperature. Centrifuge at approximately 2000 x g for 20 min. Analyze the serum immediately or aliquot and store frozen at -20° C.
   - **Plasma:** Collect plasma using heparin/EDTA/citrate as an anticoagulant. Centrifuge for 20 min at 2000 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 20.000 pg/ml mouse P-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 0.2 ml of the mixed 20,000 pg/ml P-selectin solution to the eppendorf tube containing 0.8 ml diluent buffer and mix to make a 4000 pg/ml P-selectin solution.
6. Label 6 eppendorf tubes with the mouse P-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 P-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 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).
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 mouse P-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.